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DETERMINATION OF THE PHYSIOLOGICAL ROLE OF THE β-LACTAMASE INHIBITORY PROTEIN (BLIP) IN *STREPTOMYCES CLAVULIGERUS*

by

WENDY THAI ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DETERMINATION OF THE PHYSIOLOGICAL ROLE OF β-LACTAMASE INHIBITORY PROTEIN (BLIP) IN *STREPTOMYCES CLAVULIGERUS* submitted by Wendy Thai in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Streptomyces clavuligerus is a Gram positive soil bacterium that produces four clavam compounds having antifungal activities; two antibiotics, penicillin N and cephamycin C; and two β-lactamase inhibitors, clavulanic acid and BLIP (β-lactamase inhibitory protein). Clavulanic acid is used clinically with β-lactam antibiotics to combat infections that would otherwise be resistant to existing β-lactam antibiotics by the production of β-lactamases. BLIP has an odd spectrum of activity and may have an alternative function in the physiology of *S. clavuligerus*. Four approaches were undertaken to determine the physiological role of BLIP and what importance β-lactamase inhibitors have in *S. clavuligerus* biology.

The *bli* gene was disrupted by inserting a thiostrepton resistance gene into the unique *Apa* I site located in the *bli* open reading frame. The disrupted *bli* gene was introduced into two *S. clavuligerus* strains, wild type *S. clavuligerus* and the clavulanic acid nonproducer, *claR*, to generate a *bli* single mutant and a *bli/claR* double mutant respectively. The BLIP nonproducing mutants did not show aberrant growth characteristics or morphology. Sporulation was not affected. Cephamycin and clavulanic acid production were comparable to the wild type strain. Production of clavam-2-carboxylate and hydroxymethyl clavam showed a transient increase in 72 hour cultures of both the *bli* and *bli/claR* mutants. Production of hydroxymethyl clavam was also elevated in 72 and 96 hour cultures of the *bli* mutant compared to wild type *S. clavuligerus*, while no difference was detected in the *bli/claR* mutant compared to the *claR* mutant.

Genetic mapping showed that the *bli* gene is located within 30 kilobases downstream of clavam biosynthetic genes. Sequencing results showed that an ABC transporter gene

was immediately downstream of *bli*. Immediately upstream of *bli* was an open reading frame, *bliup1*, which showed no similarity to any known protein. No open reading frame was found within 1 kilobase upstream of *bliup1*.

Nutritional studies showed that BLIP was produced at high levels in trypticase-soy-starch broth. Levels of BLIP were detectable but much lower in the minimal media starch-asparagine and glycerol-sucrose-proline-glutamate. Although large amounts of BLIP were produced in trypticase-soy-starch broth, no BLIP was seen in the complex soy medium despite its similarity to trypticase-soy-starch broth. No correlation was seen between the production of BLIP and of the other β-lactams when grown in different media.

Go, wondrous creature! mount where science guides,
Go, measure earth, weigh air, and state the tides;
Instruct the planets in what orbs to run,
Correct old time, and regulate the sun;
Go, soar with Plato to the'empyreal sphere,
To the first good, first perfect, and first fair;
Or tread the mazy round his followers trod,
And quitting sense call imitating God;
As Eastern priests in giddy circles run,
And turn their heads to imitate the sun.
Go, teach Eternal Wisdom how to rule---
Then drop into thyself, and be a fool!

...

Trace science then, with modesty thy guide;
First strip off all her equipage of pride;
Deduct but what is vanity, or dress,
Or learning's luxury, or idleness;
Or tricks to show the stretch of human brain,
Mere curious pleasure, or ingenious pain;
Expurge the whole, or lop th'excrescent parts
Of all our vices have created arts;
Then see how little the remaining sum,
Which served the past, and must the times to come!

Alexander Pope,
AN ESSAY ON MAN, Epistle II:
Of the Nature and State of Man with Respect to Himself, as an Individual

Cease then, nor order imperfection name:
Our proper bliss depends on what we blame.
Know thy own point: this kind, this due degree
Of blindness, weakness, Heaven bestows on thee.
Submit.---In this, or any other sphere,
Secure to be as blessed as thou canst bear:
Safe in the hand of one disposing Power,
Or in the natal, or the mortal hour.
All Nature is but art, unknown to thee;
All chance, direction, which thou canst not see;
All discord, harmony not understood;
All partial evil, universal good:
And, spite of pride, in erring reason's spite,
One truth is clear, Whatever is, is right.

Alexander Pope
AN ESSAY ON MAN, Epistle I:
Of the Nature and State of Man with Respect to the Universe .

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LIST OF ABBREVIATIONS

A	alanine
ABC	ATP-binding cassette
ACV	γ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	ACV synthetase
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
BLIP	β-lactamase inhibitory protein
blidwn	*bli* downstream open reading frame
bliup	*bli* upstream open reading frame
BLP	BLIP-like protein
BSA	Bovine serum albumin
CAS	Clavaminate synthase
ccaR	Cephamycin clavulanic acid regulator
CDO	*cas1* downstream orf
C-N	Carbon-Nitrogen bond
CUO	*cas1* upstream orf
D	Aspartic acid
DACS	Deacetylcephalosporin C
DAOC	Deacetoxycephalosporin C
DAOCS	DAOC synthase
DD	D-alanine-D-alanyl
dCTPs	deoxycytosine triphosphates
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
dNTPs	deoxynucleoside triphosphates
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
ESS	*Escherichia coli* super sensitive
G	Glycine
GSPG	Glycerol-sucrose-proline-glutamic acid
H	Histidine

HPLC	High performance liquid chromatography
hygR	hygromycin resistance
IgG-POD	Peroxidase labelled Immunoglobulin
IPN	Isopenicillin N
IPNE	IPN epimerase
IPNS	IPN synthase
IPTG	Isopropylthio-β-D-galactoside
ISP-3	International *Streptomyces* project-medium- 3
K	Lysine
kb	kilobase
KDa	Kilodalton
LB	Luria-Bertani
LL-DAP	LL-diaminopimelic acid
MOPS	3-[N-Morpholino]propanesulfonic acid
MSD	Membrane spanning domain
MYM	Maltose-yeast extract-malt extract
N	Asparagine
(N)AcG	N-acetyl glucosamine
(N)AcM	N-acetyl muramic acid
OD	Optical density
ORF	Open reading frame
P	Protoplast buffer
PAGE	Polyacrylamide gel electrophoresis
PBPs	Penicillin binding proteins
PCA	Perchloric acid
PCG	Percent CG
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Polyvinylidene fluoride
rpm	revolutions per minute
S	Serine
SA	Starch-asparagine
SDS	Sodium dodecyl sulfate
SG	Starch-glutamic acid

SNA	Soft nutrient agar
SSI	*Streptomyces* subtilisin inhibitor
SSPE	Standard saline-phosphate-EDTA
T	Threonine
T buffer	Transformation buffer
TBS	Tris buffered saline
TBST	Tris buffered saline-tween
TCA	Trichloroacetic acid
TE	Tris-EDTA buffer
TES	(N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid)
TRIS	Tris(hydroxylmethyl)-aminomethane
TSB	Trypticase soy broth
TSBM	Trypticase soy broth-maltose
TSBS	Trypticase soy broth-starch
tsr	Thiostrepton resistance
TSS	Transformation and storage solution
v/v	Volume per volume
w/v	Weight per volume
X	unknown amino acid
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Y	Tyrosine
YEME	Yeast extract-malt extract
YEMEM	Yeast extract-malt extract-maltose

I. INTRODUCTION

Streptomyces, one of the five genera of the family Streptomycetaceae, consists mainly of soil bacteria distinguished from other genera by the presence of LL-DAP and glycine interpeptide bridges in their cell wall structure (Goodfellow and Cross., 1984). They are Gram positive soil bacteria that have been studied extensively because of their complex life cycle and the vast array of secondary metabolites they produce. *Streptomyces* is the most common genus of this family and accounts for the largest group of bacteria in the soil (Ogawara, 1975) and produces the greatest variety in structure and number of antibiotics (Crueger and Crueger, 1990). *Streptomyces* species grow on insoluble organic remains of plants and fungi by the formation of branched subsurface mats of mycelia. The subsequent differentiation into aerial mycelia sets the stage for formation of dispersible spores which allows for an otherwise anchored organism to colonize a new environment. Coincident with differentiation is the production of biologically active secondary metabolites consisting of antibiotics, antifungals, herbicides and antitumor antibiotics among others (Crueger *et al.*, 1990). Members of the *Streptomyces* genus are also unique in their production of a vast array of exocellular degradative enzymes such as agarase, endoglycosidase H, α-amylase and proteases, all of which account for their nutritional versatility. Along with the production of exocellular enzymes, *Streptomyces* species produce a variety of enzyme inhibitors including inhibitors for α-amylase and proteases (Strickler *et al.*, 1992).

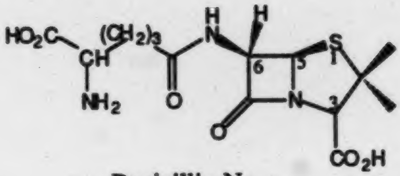
One *Streptomyces* species that has been the subject of extensive research in the last thirty years is *Streptomyces clavuligerus*. *S. clavuligerus* was isolated in 1971 from a South American soil sample (Higgens and Kastner, 1971). Like other members of the genus, *S. clavuligerus* undergoes a morphologically complex life cycle which involves growth initially as filamentous mycelia followed by differentiation into dispersible spores. The species *clavuligerus*, derived from the Latin *clavula* for little club and *igerus* meaning bearing, describes a bacterium with characteristic club-like side branches that was notable for the production of two new antibiotics, penicillin N and cephamycin C, at the time of isolation and characterization (Higgins and Kastner, 1971). Today, *S. clavuligerus* is known to produce a variety of antibiotics and extracellular products including β–lactams having antimicrobial activity and antifungal activity, two exocellular proteases and two characterized inhibitors of β–lactamase. β–lactamase is a β–lactam antibiotic degradative enzyme, production of which is the primary mechanism of clinical resistance to β–lactam antibiotics seen in pathogenic bacteria. In addition to β-lactam compounds, *S. clavuligerus* also produces two other non-β-lactam type antibiotics. These are holomycin, a pyrrothine-type antibiotic having activity against both Gram positive and Gram negative bacteria and a tunicamycin-like compound, a glucosamine-containing antibiotic having activity against Gram positive bacteria, fungi and viruses (Kenig and Reading, 1979).

The major β-lactam compounds produced by *S. clavuligerus* are shown in Figure 1. All β-lactams have the planar four membered β-lactam ring structure which is the active center of these compounds. Those having antibiotic activity include cephamycin C and penicillin N. Although the active center of β-lactams is the amide bond in the β-lactam ring, their spectrum of activity depends greatly on the second ring structure as well as on side chains. Cephalosporins and cephamycins differ from penicillins in that the second sulfur containing ring structure is a six membered dihydrothiazine ring in contrast to the five membered thiazolidine ring of penicillins. The cephalosporins have a broader spectrum of activity than do penicillins while cephamycins have greater intrinsic resistance to β-lactamases. The five compounds shown in Figure 1 B are collectively known as clavams. Clavams differ from the penicillins, cephalosporins and cephamycins in that the sulfur moiety in the second ring is replaced by an oxygen (oxazolidine ring) and the 6-acyl amino group is absent (Iwata-Reuyl and Townsend, 1992 and Sutherland, 1991). *S. clavuligerus* is believed to produce four clavam compounds with the same β-lactam/oxazolidine bicyclic clavam ring structure but different side chains at the C-2S position. These include the clavam-2-carboxylate, hydroxymethyl clavam, alanyl clavam and 2-formyloxymethyl clavam. These clavams have weak antibacterial activity and are known to be effective antifungal agents (Pruess and Kellett, 1983). In addition to these, *S. clavuligerus* also produces an atypical clavam known as clavulanic acid. Clavulanic acid has the characteristic oxazolidine ring structure of the clavams, but it has the C-5R stereochemistry as opposed to the C-5S stereochemistry of the other clavams (Baldwin *et al.*, 1994) and like the penicillins, a carboxyl group occurs at the C-3 position. Although possessing low antibacterial activity, clavulanic acid has become a clinically important product of *S. clavuligerus* due to its potent inhibitory activity against a large number of β-lactamases (Baggaley *et al.*, 1997).

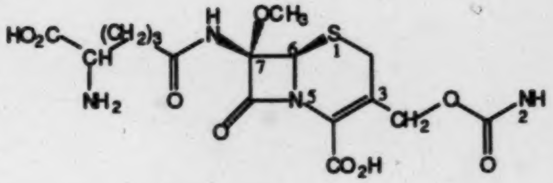
β-lactam antibiotics such as penicillin N and cephamycin C function as bactericidal compounds by inhibiting cell wall biosynthetic enzymes known as transpeptidases.

Figure 1. β-lactam products of *Streptomyces clavuligerus*. A) β-lactam products having antimicrobial activities include penicillin N and cephamycin C. B) The clavams, β-lactams which have antifungal activity include clavam-2-carboxylate, hydroxymethyl clavam, alanyl clavam and formyl-oxymethyl clavam and the antipodal clavam, clavulanic acid, the clinically important β-lactamase inhibitor.

A.

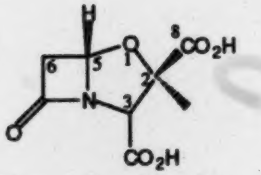

HO2C
CH
NH2
(CH2)3
O
H
N
H
6
5
S
1
3
N
O
CO2H

Penicillin N

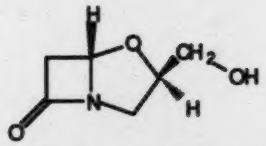

HO2C
CH
NH2
(CH2)3
O
H
N
OCH3
7
6
S
1
N
5
3
O
CO2H
CH2
O
NH
2
O

Cephamycin C

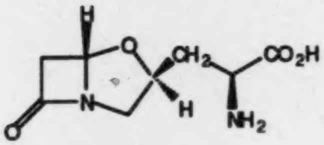
B.


H
6
5
O
1
8
CO2H
2
3
N
O
CO2H

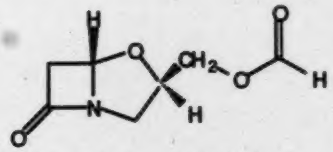
Clavam-2-carboxylate


H
O
CH2
OH
N
H
O

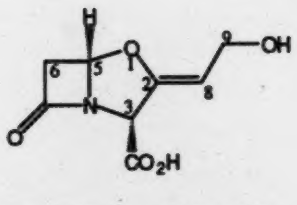
Hydroxymethyl clavam


H
O
CH2
CO2H
N
H
NH2
O

Alanyl clavam


H
O
CH2
O
O
H
N
H
O

Formyl-oxymethyl clavam


H
6
5
O
1
9
OH
2
8
3
N
O
CO2H

Clavulanic acid (C-5R)

Bacterial cell wall peptidoglycan consists of linear strands of glycan crosslinked by short peptides. The glycan chain of peptidoglycan consists of alternating units of N-acetylglucosamine (N-AcG) and N-acetylmuramic acid (N-AcM), depicted in Figure 2 A. The N-acetylglucosamine-N-acetylmuramyl-peptide subunit of the peptidoglycan is synthesized intracellularly, transported across the cytoplasmic membrane by a lipid carrier and assembled exocellularly in two steps (Frere *et al.*, 1992). The first is the polymerization of the glycan chain by a transglycosylation reaction between a disaccharide unit and the growing glycan chain and second, the formation of peptide crosslinks between adjacent glycan chains by transpeptidation. This latter step of strand assembly is catalyzed by enzymes known as transpeptidases (Figure 2 B).

Transpeptidases are also called penicillin binding proteins (PBPs) because of their affinity for penicillins. PBPs are serine active enzymes. The serine residues at their active sites react with the D-alanine-D-alanyl terminus of the pentapeptide to form a serine ester-linked acyl-enzyme intermediate. When the acyl-enzyme intermediate reacts with an acceptor pentapeptide on a separate glycan chain, the enzyme is released and a peptide cross link is generated between two glycan chains. β-lactam antibiotics such as penicillins compete with the D-alanine-D-alanyl terminus of pentapeptides for the active site of PBPs. The cyclic amide bonds in the β-lactam rings of β-lactams resemble the D-alanyl-D-alanine C-terminus of the pentapeptide (Figure 2 C).

In the presence of β-lactam antibiotics, the hydroxyl group of the active site serine residue of PBPs participates in a nucleophilic reaction with the C-7 carbonyl group in the β-lactam ring forming a penicilloyl enzyme intermediate (Figure 3 A). This resulting serine acyl enzyme intermediate is hydrolysed so slowly that the enzyme is effectively prevented from catalyzing subsequent transpeptidation reaction. The net result is the formation of peptidoglycan defective and osmotically fragile cells (Ghuysen, 1991).

PBPs fall into two classes, the low-Mr PBPs and the high-Mr PBPs, and their molecular weights range between 25 to 120 kDa. The low Mr-PBPs are mainly carboxypeptidases which do not play a major physiological role in cell wall biosynthesis. Carboxypeptidases catalyse proteolytic rupture of the D-alanine-D-alanyl peptide bond at the pentapeptide C-terminal of peptidoglycan precursor through formation of a serine ester-linked acyl-enzyme intermediate. The difference between carboxypeptidases and transpeptidases which catalyse the peptide crosslinking reactions in peptidoglycan biosynthesis is the nature of the acceptor group that resolves the acyl-enzyme intermediate. In carboxypeptidases/DD-peptidases, the acyl enzyme

Figure 2. Cell wall structure and biosynthesis of peptide crosslinks. A) The structure of the peptide crosslinking between two glycan chains is shown. B) Formation of the acyl enzyme intermediate occurs by a reaction between the serine hydroxyl of the enzyme with the D-ala-D-ala peptide bond of the peptidoglycan precursor resulting in release of the terminal D-alanine (steps 1 and 2). A second reaction of the acyl intermediate with the second peptidoglycan precursor (step 3) resolves the acyl enzyme intermediate (step 4) and a new peptide crosslink shown in bold is formed.

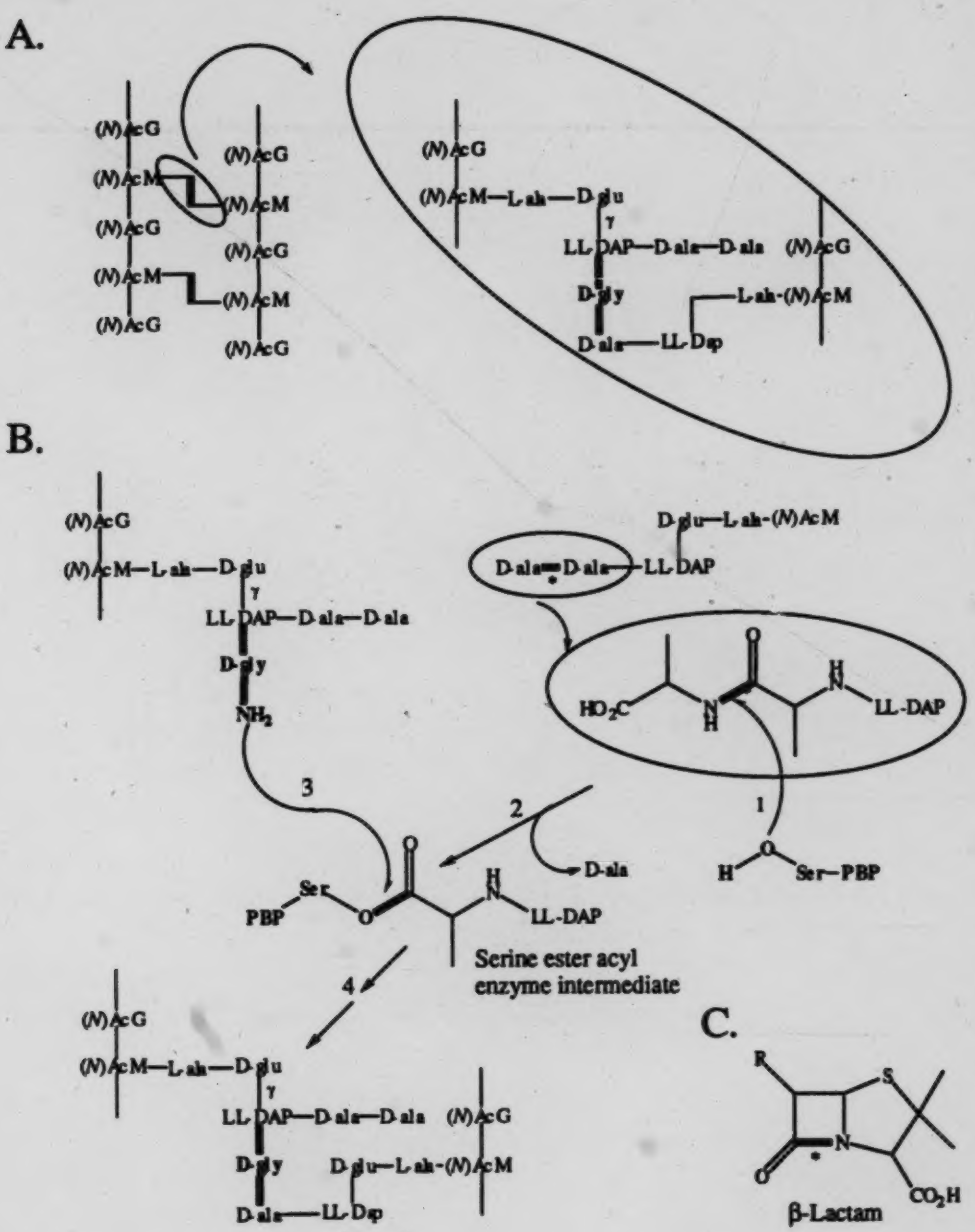
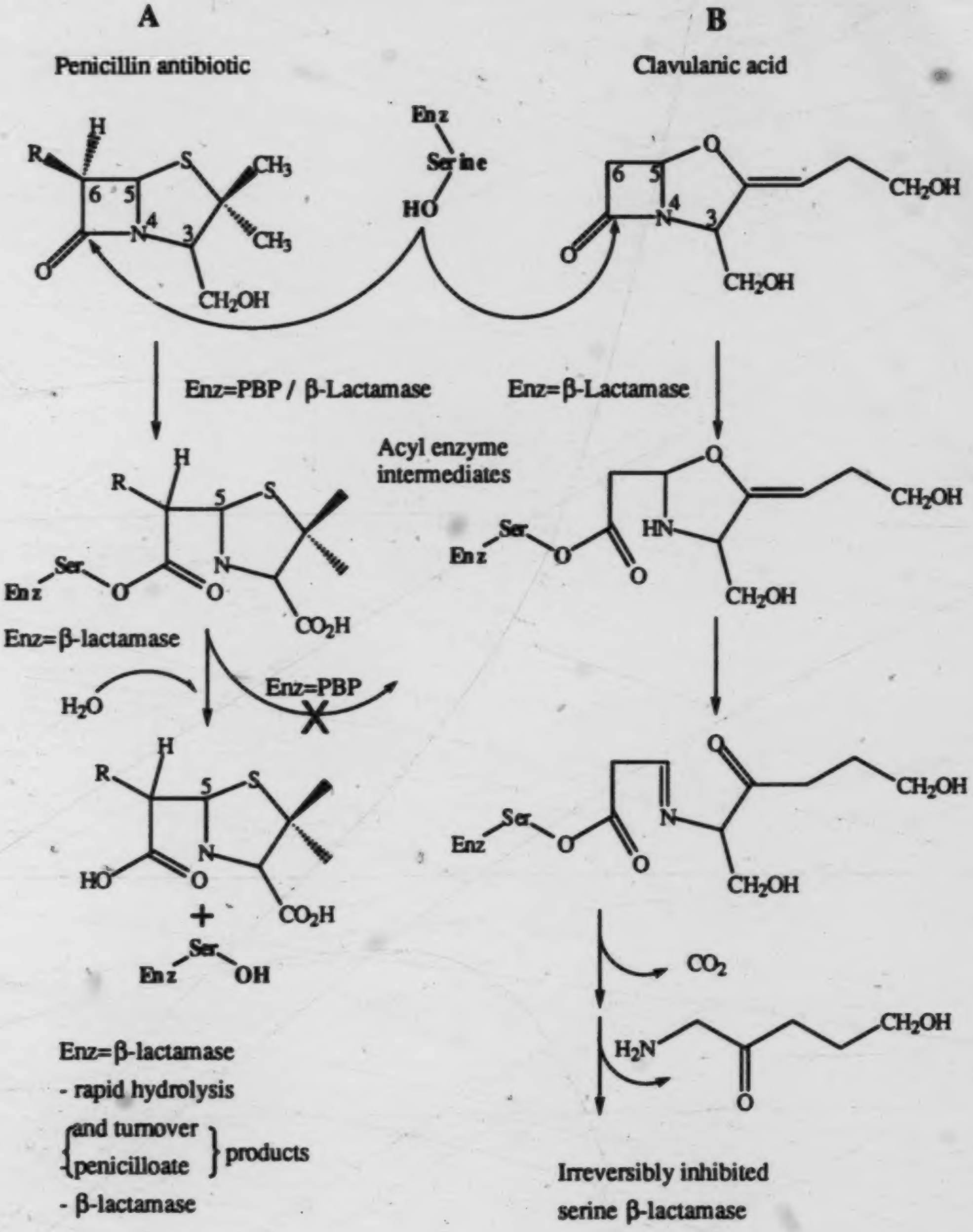
A.
(N)AcG
(N)AcM
(N)AcM—L-ala—D-glu
γ
LL-DAP—D-ala—D-ala
D-gly
D-ala—LL-Dap
L-ala-(N)AcM
B.
NH2
D-glu—L-ala-(N)AcM
D-ala—D-ala—LL-DAP
O
HO2C
H
N
LL-DAP
1
2
3
4
D-ala
H
Ser—PBP
PBP
Ser
Serine ester acyl
enzyme intermediate
C.
R
S
N
O
CO2H
β-Lactam

Figure 3. Interactions of penicillin antibiotic and clavulanic acid with the serine enzymes β-lactamase and PBPs. A) Interaction of penicillin with PBP or β-lactamase. Penicilloyl-PBP intermediate is very slowly resolved and the PBP is effectively removed from further cell wall biosynthetic reactions. The penicilloyl-β-lactamase intermediate is hydrolysed resulting in turnover of β-lactamase and cleavage of the β-lactam ring to give penicilloic acid. B) Irreversable interaction of clavulanic acid with β-lactamase. The acyl enzyme intermediate undergoes a series of reactions resulting in eventual complete breakdown of the ring structures of clavulanic acid.


A
Penicillin antibiotic
B
Clavulanic acid
Enz
Serine
HO
R
H
S
CH3
6
5
4
3
N
O
CH2OH
Enz=PBP / β-Lactamase
Enz=β-Lactamase
Acyl enzyme intermediates
Ser
Enz
HN
CO2H
Enz=β-lactamase
H2O
Enz=PBP
HO
OH
CO2
H2N

Enz=β-lactamase
- rapid hydrolysis
and turnover } products
penicilloate
- β-lactamase

Irreversibly inhibited
serine β-lactamase

intermediate is hydrolysed. In transpeptidases, the acceptor group with greatest affinity for the acyl-transpeptidase intermediate is a NH_2-R group that, in cross linking reactions of peptidoglycan biosynthesis, is the acceptor pentapeptide (Ghuysen, 1991). Although the 27 kDa *Streptomyces* K15 DD-transpeptidase is an exception (Palomeque-Messia *et al.*, 1991), transpeptidation reactions are generally catalysed by high-Mr PBPs (Mr > 50 kDa), the kind important for cell morphology and inhibition of which leads to aberrant morphology and cell death (Adam *et al.*, 1991). These high-Mr PBPs have two functional domains: an amino terminal domain containing a membrane anchor region is fused to a carboxy terminal domain which is sensitive to β-lactam antibiotics. Although in general, the mechanism of transpeptidation by high-Mr PBPs is not clearly understood, in the *E. coli* high-Mr PBP 1b specifically, the N-terminal domain contains a region which catalyses transglycosylation while the C-terminal domain catalyses transpeptidation and is the penicillin sensitive domain.

All low-Mr PBPs and the penicillin binding domains of the high-Mr PBPs are characterized by four conserved amino acid signatures in their primary sequence. These are the tetrad serine-X-X-lysine (S*-X-X-K), the triad serine or tyrosine-X-asparagine (S/Y-X-N), a peptide segment containing two dicarboxylic amino acids (D~E) and the triad lysine or histidine-threonine or serine-glycine (K/H-T/S-G). These four conserved motifs are brought together to form the walls of the active site of the enzyme by the folding of the polypeptide chain. The S* is the enzyme active site serine residue whose hydroxyl group partakes in the nucleophilic reaction with the C-7 carboxyl group of the amide bond of the β-lactam antibiotic.

Little recent research has been done on the PBPs of *S. clavuligerus*. In 1980, Ogawara compared PBPs of two antibiotic producers, *S. olivaceus* and *S. clavuligerus* and a β-lactam non producer, *S. cacaoi*. He reported the detection of membrane bound, exocellular and soluble PBPs of *S. clavuligerus* (Mr 36-83 kDa), *S. olivaceus* (Mr 47-72) and *S. cacaoi* (Mr 47-105) by binding to ^{14}C-labelled benzylpenicillin. His study showed that fewer PBPs were seen in the two β-lactam producers *S. clavuligerus* and *S. olivaceus* than in *S. cacaoi*, the non producer. The PBPs of *S. clavuligerus* and *S. olivaceus* do not bind to clavulanic acid. They also have a lower affinity for benzylpenicillin than those of the β-lactam nonproducer *S. cacaoi* (Ogawara and Horikawa, 1980). Furthermore, the PBP patterns of the β-lactam producers change significantly throughout the growth phase of the organism, more PBPs were detected in early logarithmic cultures than in older cultures, while those of *S. cacaoi*, were similar throughout the growth cycle (Horikawa *et al.*, 1980). Nakazawa *et al.* (1981), proposed that the PBPs in *Streptomyces* be classified into two groups. Those from β-lactam nonproducers were similar throughout the growth phase and alike among different nonproducing species. They were also present in greater numbers and have greater affinity for benzylpenicillin. PBPs from β-lactam producers were fewer in number and have much lower affinities for benzylpenicillin. They also tend to change during different stages of growth and vary from species to species.

Recent work on *Streptomyces* PBPs in general included the use of the low-Mr DD-carboxypeptidase of *Streptomyces* R61 (Bourguignon-Bellefroid *et al.*, 1992; Wilkin *et al.*, 1994) and the unusual DD-transpeptidase of *Streptomyces* K15 (Palomeque-Messia *et al.*, 1991) as model systems for further understanding the mechanism of transpeptidation. PBPs of *S. griseus* protoplasts (Barabas *et al.*, 1988) and those involved in aerial mycelia formation (Hao and Kendrick, 1998) have also been the subject of recent research although not much more will be discussed here.

It is believed that PBPs gave rise to β-lactamases through divergent evolution. β-lactamases are thought to have evolved from PBPs initially for protection from β-lactam antibiotics of other bacteria (Ghuysen, 1991) or for protection of β-lactam producers from their own products (Ogawara, 1980). The term penicillinase was first used in 1940 by Abraham and Chain to describe an enzyme detected in crushed cells of a strain of *E. coli* that was able to inactivate penicillin. Four years later, Kirby (1944), reported the discovery that resistant strains of *Staphylococcus aureus* possess an enzyme that inactivated penicillin. Today, the production of β-lactamases is one of the major clinical mechanism of resistance to β-lactam antibiotics among pathogenic bacteria. β-lactamases can be found in both Gram positive and Gram negative bacteria. They can be chromosomally or plasmid encoded and are found in both antibiotic producers and non producers alike. In terms of their mechanism of action, there are two types of enzymes. The first is the EDTA sensitive and Zn^{2+} dependent β-lactamases while the second group consist of the PBP-like serine active enzymes.

Several classification schemes were devised for β-lactamases by Richmond and Sykes (1973), Bush (1989a, b and c) and Ambler (1980). These were based on sequence similarities, substrate preference, catalytic mechanism, and other biochemical properties. In the Ambler classification scheme (1980), β-lactamases are divided into three groups on the basis of amino acid and nucleotide sequences. Class A consists of all serine enzymes that preferentially hydrolyse penicillins. Examples of these include the TEM plasmid mediated enzymes and the *S. aureus* enzymes. Class B enzymes are metalloenzymes that require Zn^{2+} for activity. Class C are serine cephalosporinases that have no homology with Class A enzymes at their primary sequence and examples of these are the chromosomally encoded β-lactamases of *Escherichia coli* and *Pseudomonas aeruginosa*. The second classification scheme that will be discussed here is based on enzymatic, biochemical and physical properties (Bush, 1989a). In this classification scheme, group 1 includes chromosomal cephalosporinases that are generally basic, larger than 30 kilodaltons and are not inhibited by clavulanic acid. Group 2 includes Gram positive penicillinases, broad spectrum β-lactamases that will hydrolyse both penicillins and cephalosporins (TEM and SHV-1 enzymes) and tend to be smaller than 30 kilodalton, as well as β-lactamases that hydrolyse extended broad spectrum antibiotics. All of the group 2 enzymes are sensitive to clavulanic acid inhibition. All enzymes classified as belonging to class A in the Ambler classification method (1980) fall into group 2. Group 3 are the metalloenzymes that require Zn^{2+} for activity and these are not inhibited by clavulanic acid. Group 4 includes a variety of penicillinases that are not sensitive to clavulanic acid inhibition.

All serine active enzymes possess four conserved elements in their amino acid sequence that are also found in PBPs. These make up the walls of the active site of the enzyme and include the S*-X-X-K element containing the catalytic serine (S*), the S/Y-X-N element, the D/E element and the K-T/S-G triad (Frere, 1995). Three dimensional structures of several class A β-lactamases determined by X-ray crystallography showed a small all α-helical domain and an α/β domain consisting of β-sheets of five anti-parallel strands which are surrounded by α-helices (Knox, 1995). The active site is located at the junction of the all α and the α/β domains. The active site serine-70 that participates in the ester-linkage in the acyl-enzyme intermediate is located in the N terminus of the α2 helix in the all α-helical domain and this region forms the central wall of the active site cavity (Ghuysen, 1991). One of the sides of the active site cavity is formed by the second conserved element, the S(Y)-X-N motif, which connects helices α4 and α5 of the all-α domain. The fourth conserved element, K-T(S)-G, forms the other side of the cavity while the third element, the D/E motif forms what is called an omega loop that lies at the entrance of the active site (Ghuysen, 1991). The long side chain of the lysine residue in the first conserved element (K-73) lies in the active site and in conjunction with the lysine residue in conserved element 4 (K-234) participates in H-bonding with the β-lactam molecule during catalysis (Adachi *et al.*, 1991). The mechanism of action of β-lactamases on β-lactams is similar to the PBPs in that an ester-linked acyl-enzyme intermediate in which the β-lactam is covalently linked to the enzyme at the serine-70 residue is formed. Unlike the case of PBP-β-lactam interactions in which the PBP is trapped as an acyl-enzyme intermediate, the ester-linked β-lactam-β-lactamase intermediate is readily resolved by hydrolysis freeing the β-lactamase enzyme for another round of reaction (Figure 3 A). The product of this nucleophilic reaction is the penicilloic acid that no longer has biological activity (Adachi *et al.*, 1991). The kinetics of this hydrolysis step differentiates β-lactamases from PBPs as in most other respects they are identical.

The interaction of clavulanic acid with β-lactamases is analagous to the reaction between penicillins and PBPs discussed above. Clavulanic acid is described as being the atypical clavam product of *S. clavuligerus* because unlike the other four clavams, clavulanic acid has a C-5R stereochemistry and the additional carboxylate moiety in the C-3 position. Both of these differences, as well as the double bond at the C-2 position, make it a weak antimicrobial agent but a potent inhibitor of β-lactamases (Baggaley *et al.*, 1997). Some of the clavulanic acid sensitive serine-type penicillinases and cephalosporinases include the Gram positive staphylococcal β-lactamases; the chromosomally encoded β-lactamases of Gram negative bacilli such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgarus*, *Branhamella catarrhalis* and *Bacteroides fragilis;* and the TEM plasmid-mediated β-lactamases from Enterobacteriaceae, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Sutherland, 1991). The β-lactamases inhibited by clavulanic acid are all serine enzymes that fall into group 2 β-lactamases (penicillinases, cephalosporinases and broad spectrum enzymes) based on Bush's classification scheme of β-lactamases (1989b and c) or class A based on Ambler's classification scheme (1980). Clavulanic acid functions as an irreversible suicide inhibitor of β-lactamases, having high affinity for the active site of the enzyme. Studies on the mechanism of inhibition of β-lactamase by clavulanic acid have been done with the class A enzymes such as the *E. coli* TEM and the *S. aureus* β-lactamase. In both cases, the hydroxyl moiety of the active site serine undergoes a nucleophilic attack on the carbonyl group of the amide bond in clavulanic acid resulting in the formation of a serine ester-linked acyl-enzyme intermediate (Figure 3 B). Like the penicillin-PBP acyl-enzyme intermediate, the clavulanic acid-β-lactamase intermediate cannot be resolved by hydrolysis. The oxazolidine ring of clavulanic acid undergoes ring cleavage and decarboxylation eventually resulting in formation of an irreversibly inactivated enzyme and complete degradation of clavulanic acid (Baggaley *et al.*, 1997).

Recently, Perez-Llarena and Martin *et al.* (1997) reported the discovery of a β-lactamase gene (*bla*) in *S. clavuligerus*. The *bla* gene of *S. clavuligerus* was found by hybridizing genomic DNA with the *Nocardia lactamdurans* β-lactamase gene. It was heterologously expressed in *Streptomyces lividans* as β-lactamase activity could not be detected in vivo in *S. clavuligerus*. The heterologously expressed enzyme was described as having poor activity. Although sequence comparisons with other enzymes place the *S. clavuligerus* β-lactamase in Ambler's class A, its catalytic activity against benzylpenicillin was determined to be 2 to 4 orders of magnitude lower than other class A enzymes. When assayed against clavulanic acid, the *S. clavuligerus* β-lactamase was found to be sensitive to inhibition by clavulanic acid although kinetic studies showed that the inhibition is weak compared to other target enzymes. Due to its penicillin binding ability (^{3}H-benzylpenicillin) but weak hydrolysing activity, Perez-Llarena *et al.* (1997) proposed that this enzyme functions in cell wall morphogenesis. This theory is also based on their finding that the analagous class A β-lactamase in *N. lactamdurans* is involved in cell wall biosynthesis as disruption of it affects morphology (Perez-Llarena and Martin *et al.*, 1997). *N. lactamdurans* is also a penicillin N and cephamycin C producer like *S. clavuligerus* and the corresponding β-lactamase gene is found near the cephamycin biosynthetic gene cluster, as in the case of the *S.clavuligerus* β-lactamase gene. The *Nocardia* β-lactamase is extracellular but cell wall associated. It is a class A enzyme. The enzyme was demonstrated to hydrolyse penicillin G and isopenicillin N at a rate of 1.25 mg/ml/min while showing much less activity against cephalosporins and no activity against cephamycins. Its linkage to the cephamycin biosynthetic gene cluster leads to the idea that it was initially intended for protection of PBPs from the cephamycin C biosynthetic pathway intermediate penicillin N (Coque *et al.*, 1993). The hypothesis that the *S. clavuligerus* β-lactamase is involved in cell wall morphogenesis does not necessarily exclude the idea of self protection as maintaining functionally active PBPs is necessary to achieve normal cell wall structure and integrity.

The finding of a penicillin binding β-lactamase with poor penicillin hydrolytic activity in *S. clavuligerus* suggests that it is a less evolved β-lactamase than those found in pathogenic bacteria (Perez-Llamera and Liras *et al.*, 1997). This supports the idea that β-lactamases originated in β-lactam producing bacteria (Ogawara *et al.*, 1978) perhaps initially in response to selective pressure from β-lactams in the soil environment or, since *Streptomyces* β-lactamases are mostly penicillinases as opposed to cephalosporinases, for self protection against the toxic intermediate penicillin N of their cephamycin biosynthetic pathways (Coque *et al.*, 1993). Although cephamycin producers such as *S. clavuligerus*, *N. lactamdurans*, *S. griseus* and *S. cattleya* all produce β-lactamases, a large number of other *Streptomyces* species including *S. cacaoi*, *S. badius*, *S. cellulosae* and *S. fradiae* that do not produce β-lactam antibiotics also produce β-lactamases. This suggests that it is unlikely β-lactamases were solely produced for self protection against toxic intermediates of the cephamycin biosynthetic pathway.

Studies on the production of β-lactamases in a hundred different *Streptomyces* species done in the 1970s showed that three quarters of *Streptomyces* strains examined produce β-lactamases exocellularly and constitutively (Ogawara, 1975). Comparison of *Streptomyces* strains isolated in the 1970s with those isolated in the 1940s showed that the proportion of strains producing β-lactamases were similar. Furthermore, enzymatic properties such as isoelectric point, molecular weight, pH optimum and substrate specificity of the enzymes produced by older strains were like those of the newer strains. Since the older strains represented *Streptomyces* that have not been exposed to β-lactam antibiotics other than those naturally occurring in the soil, the similarity in properties and frequency showed that the production of β-lactamases by *Streptomyces* does not occur in response to antibiotic use (Ogawara *et al.*, 1978). This conclusion agrees with the finding that no correlation exists between β-lactamase production and susceptibility to benzylpenicillin, as reflected in minimum inhibitory concentrations seen in β-lactamase producing *Streptomyces* strains. As the *Streptomyces* PBPs themselves have low affinities for β-lactams (Ogawara and Horikawa, 1980), it is possible that β-lactamases were the initial resistance enzymes until low affinity PBPs evolved in the *Streptomyces* β-lactam producers. Perhaps some of the β-lactam binding enzymes considered as β-lactamases which are seen in *Streptomyces* today are types of PBPs involved in morphogenesis as the difference between β-lactamases and PBPs lies mainly in the rate of hydrolysis of the acyl-enzyme intermediate which is more a factor of the presence of certain amino acid residues than in large tertiary structural and mechanistic differences (Palomeque-Messia *et al.*, 1991).

In addition to clavulanic acid, *S. clavuligerus* also produces a second β-lactamase inhibitor. BLIP, β-lactamase inhibitory protein, is an exocellular protein of molecular weight 17.5 kilodaltons. The amino acid sequence of BLIP shows that it has a 36 amino acid signal sequence (Doran *et al.*, 1990) and is an internal dimer consisting of a tandem imperfect 76 amino acid repeat (Strynadka *et al.*, 1994). Unlike clavulanic acid which is a suicide inhibitor of β-lactamase, BLIP inhibits TEM β-lactamase in a non covalent fashion (Doran *et al.*, 1990) such that neither enzyme nor inhibitor is changed in the interaction. The BLIP-β-lactamase complex can be resolved by SDS-PAGE into intact enzyme and inhibitor. X-ray crystallography of BLIP shows that it is a relatively flat molecule, each domain of the protein consisting of a helix-loop-helix motif that packs against four anti-parallel β-strands (Strynadka *et al.*, 1996). The position and curvature of the eight β-sheets of the two domains form a slightly concave sheet. In the TEM-BLIP interaction, the concave fold of BLIP, containing mostly polar uncharged residues (Ser, Thr, Tyr), wraps around a protruding negatively charged loop/helix of TEM that lies immediately adjacent to the TEM active site cavity. A second region of interaction occurs between the active site cavity of TEM and a β-hairpin turn between β-strands 2 and 3 of domain 1 of BLIP. In this interaction, the β-hairpin of BLIP inserts into the active site of TEM such that the Asp49 residue of the hairpin turn forms four H-bonds with conserved catalytic residues in the walls of the active site. These interactions between BLIP and TEM result in inhibition kinetics with a Ki of 0.6 nM (Strynadka *et al.*, 1994).

Unlike clavulanic acid, the spectrum of activity of BLIP is somewhat unusual. Both BLIP and clavulanic acid have no inhibitory activity against the Zn^{2+} dependent metalloenzymes (Strynadka *et al.*, 1994). Within the group 1 chromosomally encoded β-lactamases, BLIP has no effect on some but enhances the activity of others such as the *Escherichia coli* ampicillin C enzyme. Of the β-lactamases within the class 2 category, BLIP has strong inhibitory activity against group 2b enzymes which include the *E. coli* TEM β–lactamases and variable activity against group 2a enzymes, inhibiting the *S. aureus* 853 and the *Bacillus cereus* ATCC 1-27348 β-lactamases, stimulating the *S. aureus* 157 enzyme while having no effect on the β-lactamase in *B. cereus* NCIB 8933. BLIP was found to have an inhibitory effect on the *S. clavuligerus* β-lactamase (Perez-Llarena and Martin *et al.*, 1997) although inhibition of this β-lactamase is a hundred fold weaker (Ki=5 μM) (Perez-Llarena and Martin *et al.*, 1997) than that of TEM (Ki=0.6 nM) (Strynadka *et al.*, 1994).

Little else is known about the nature of the function of BLIP beyond its β-lactamase inhibiting ability and kinetics. Perez-Llarena and Liras *et al.*, (1997) reported the presence of a gene in the genome of *S. clavuligerus* that encodes a protein, BLP for

BLIP-like protein, whose amino acid sequence shows significant homology to BLIP having 29.2 % identity and an additional 27.6 % functionally conserved residues (values smaller than was obtained with GCG/netBLAST search which reported 33.8 % identity and 42.2 % similarity at the amino acid level). The alignment of mature BLIP and BLP by GCG/netBLAST is shown in Figure 4. The deduced BLP protein is 182 amino acids containing an N-terminal leader sequence of 28 amino acids. Computer prediction of the tertiary structure of BLP also shows significant similarity between the tertiary structure of BLIP, which has been determined by X-ray crystallography (Strynadka *et al.*, 1994), and BLP (Strynadka, personal communication). Interestingly, the BLP gene, *blp*, is located in the cephamycin C biosynthetic gene cluster. It does not appear to be required for production of β-lactam antibiotics however since deletion of the *blp* gene has no effect on cephamycin C production in *S. clavuligerus* (D. Alexander, 1998).

β-lactamase inhibitory proteins have also been detected in the culture supernatants of *Streptomyces exfoliatus* SMF19 (Kim and Lee, 1994). BLIP-I and BLIP-II of *S. exfoliatus* are 48 and 33 kDa, larger than the *S. clavuligerus* BLIP. Beyond initial purification and kinetic studies, nothing is known about the function of these proteins as well. One other proteinaceous inhibitor from *Streptomyces gedanensis* showing activity against the *Staphylococcus aureus* β-lactamase has been reported by Hata *et al.*, in 1972, however no further characterization of this activity has been done. It has been proposed by Doran *et al.* (1990) that the *S. clavuligerus* BLIP functions as a regulator of cell wall growth or morphogenesis. Perez-Llarena and Martin *et al.* (1997) suggest that perhaps the *S. clavuligerus* β-lactamase functions in cell wall synthesis and morphology, its activity in turn being regulated by BLIP and the β-lactam clavulanic acid.

In addition to proteinaceous β-lactamase inhibitors such as those discussed above, a large number of exocellular proteinaceous inhibitors of serine proteases have been detected in *Streptomyces*. The first proteinaceous inhibitor isolated from bacteria (Satow *et al.*, 1973) was SSI, Streptomyces subtilisin inhibitor, produced by *Streptomyces albogriseolus* S-3252. SSI is an exocellular protein that functions as a dimer of two identical subunits each subunit being 11.4 kilodalton (Obata et al, 1989). A large number of protease inhibitors belonging to the SSI family (dimeric proteins consisting of two identical subunits each composed of approximately 100 amino acids that strongly inhibit subtilisin by forming an inhibitor enzyme complex in a 2:2 molar

Figure 4. Alignment of the amino acid sequence of the mature BLIP and BLP by GCG/netBLAST. Numbers indicate amino acid residues. Vertical bars indicate identical amino acids; colons indicate similar amino acids. Dots between amino acid letters represent gaps introduced to maximize alignment.

```
             .         .         .         .         .
  1 ............................AGVMTGAKFTQIQFGMTRQQVL 22
                                    |  :: .||||| |  |
  1 MVKKTWRSALALTAAAGAVLATATSAHAYTGFTPERYNKIQFGMDRTLVW 50
             .         .         .         .         .
 23 DIAGAENCETGGSFGDSIHCRGHAAGDYYAYATFGFTSAAADAKVDSKSQ 72
     :|||:  :.     : | |  .   |:|      | .||   |.  | |
 51 QLAGAD..QSCSDQVERIICYNNP..DHYGPQGHFFFNAA..DKLIHKRQ 94
             .         .         .         .         .
 73 EKLLAPSAPTLTLAKFNQVTVGMTRAQVLATVGQGSCTTWSEYYPAYPST 122
     .|     ||: || :|.   ||| ||  | |   .|.  .| || :|.|
 95 MELFPAPKPTMRLATYNKTQTGMTEAQFWAAVPSDTCSALAEQYPNWPAT 144
             .         .         .         .
123 AGVTLSLSCFDVDGYSSTGFYRGSAHLWFTDGVLQGKRQWDLVZ 166
     |      |      |    :  ||:  |||| |  : |  |
145 NGNLREYVC.....PSKAERFAPSAYFTFTDGKLTSRSQSQLP. 182
```

ratio) have since been found in *Streptomyces* species (Taguchi *et al.*, 1993) including plasminostreptin from *S. antifibrinolyticus* (Ueda *et al.*, 1992); API-2c', alkaline protease inhibitor, from *S. griseoincarnatus* (Ueda *et al.*, 1992); SLPI from *S. lividans* (Ueda *et al.*, 1992) and STI2 of *S. longisporus* (Strickler *et al.*, 1992). In addition to these, Taguchi *et al.*, (1993) assayed for the presence of SSI-like protease inhibitors, in the supernatants of fifty *Streptomyces* species and found that 78 % of the strains screened showed Subtilisin BPN' inhibitory activity (Taguchi *et al.*, 1993). BLIP does not show homology to SSI nor to SSI-like proteins. To date β-lactamase inhibitory proteins have been characterized from only two *Streptomyces* species, *S. clavuligerus* and *S. exfoliatus*, and these are significantly larger than SSI-like proteins. Furthermore, the *S. clavuligerus* BLIP and those of *S. exfoliatus* do not show significant homology with each other. SSI and SSI-like inhibitors from different *Streptomyces* species tend to have monomeric masses of approximately 10 kDa and show upwards of 65 % homology with each other at the amino acid level (Strickler at la., 1992 and Ueda *et al.*, 1992).

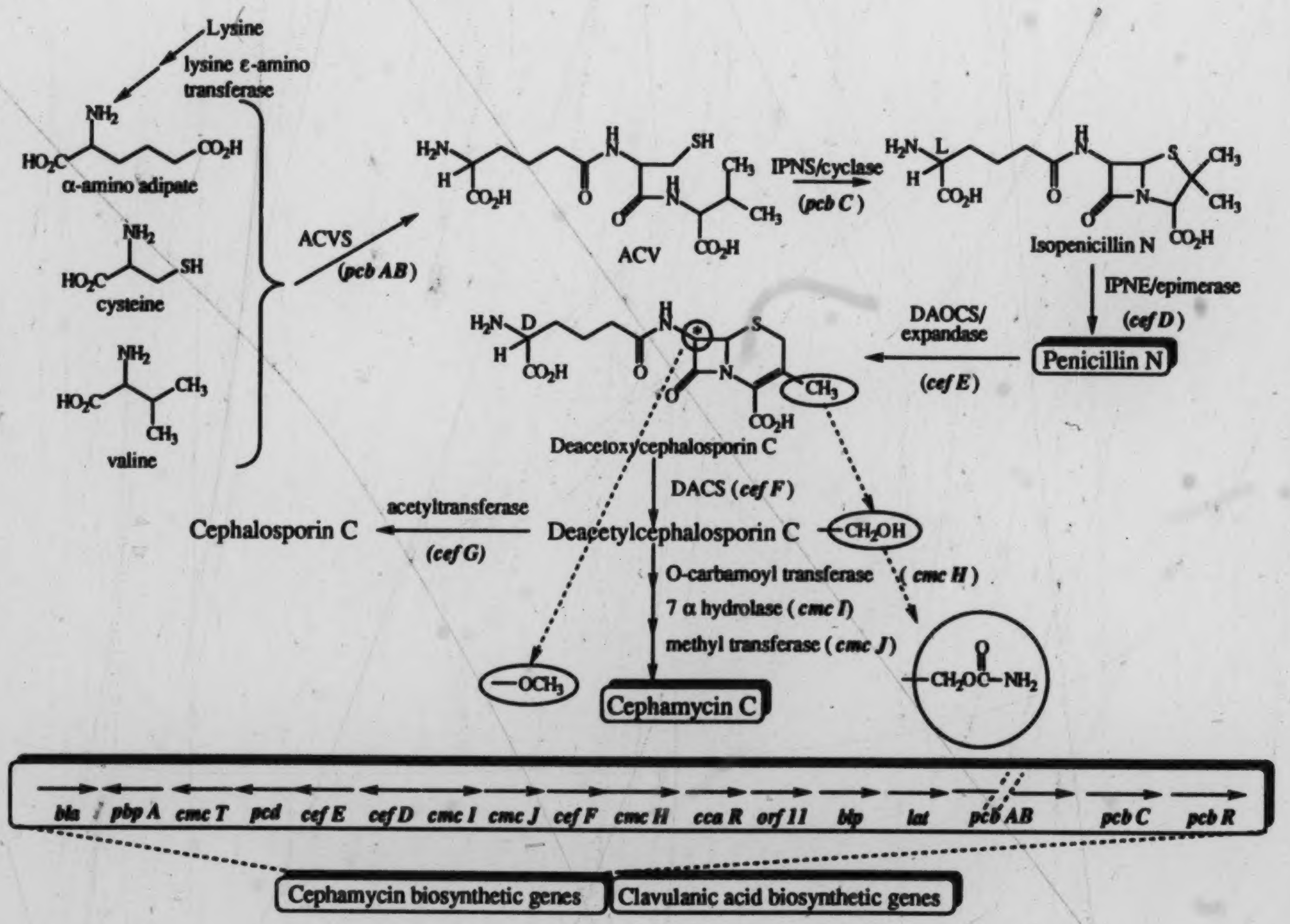
Subtilisin, the target of SSI, is an exocellular protease produced by *Bacillus amyloliquefaciens*. Proteases are grouped into four classes based on their mechanism of action. These are the acid, metallo, serine and thiol proteases. The serine proteases include the chymotrypsin-like, trypsin-like and the alkaline serine proteases of which subtilisin is an example. All serine proteases have at the active site a serine residue that participates in the catalytic reaction.

S. clavuligerus also produces exocellular proteases. In 1980, Jeffries and Buckley reported the discovery of a serine trypsin-like enzymatic activity in *S. clavuligerus* cultures grown in tryptic digest of casein. A metalloenzyme sensitive to the addition of EDTA was also found to be coproduced with the serine trypsin-like enzyme (Buckley and Jeffries, 1981). Recent work on exocellular proteases of *S. clavuligerus* involved studying the nutritional requirements for protease production (Bascaran *et al.*, 1990 and Porto *et al.*, 1996) but little else is known of the nature of the enzymes.

Since the discovery that *S. clavuligerus* produces β-lactam antibiotics in 1971 (Higgens and Kastner, 1971) and the discovery of clavulanic acid in 1976 (Brown *et al.*, 1976), research on *S. clavuligerus* has focused on elucidating the pathways involved in production of these compounds and the genes that encode the enzymes involved. Genetic research was approached with the general goal of understanding the regulation of the expression of β-lactam biosynthetic genes in order to enhance production of these compounds. Although penicillin N, cephamycin C, clavulanic acid and the clavams are all β-lactam type metabolites, at least two distinct biosynthetic pathways with different amino acid precursors exist for their production. The best understood pathway is that involved in penicillin N and cephamycin C production in *S. clavuligerus*. Although the complete biosynthetic pathway for clavulanic acid has not been determined, several intermediates, enzymes and the locations of the corresponding genes have been characterized.

The common biosynthetic pathway of penicillin N and cephamycin C is shown in Figure 5. The locations of the genes encoding each enzyme in *S. clavuligerus* are shown below the pathway. The starting amino acid precursors of the penicillin N and cephamycin C pathway are valine, cysteine and α-aminoadipate. In *S. clavuligerus*, α-aminoadipate is synthesized from lysine by lysine ε-aminotransferase, the *lat* gene product and the first enzyme in the pathway. Condensation of the three amino acid precursors to give the linear tripeptide γ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) is catalyzed by a later enzyme in the pathway, ACV synthetase, encoded by the *pcbAB* gene. The first bicyclic intermediate containing the β-lactam ring is isopenicillin N resulting from the cyclization of ACV by IPNS, isopenicillin N synthase, a product of the *pcbC* gene. Isopenicillin N epimerase, IPNE, (the *cefD* gene product), then changes the stereochemistry of the α-aminoadipate side chain from L to D giving penicillin N. The thiazolidine ring of penicillin N is then expanded to the dihydrothiazine ring of cephalosporins and cephamycins by the insertion of one of the C-2 methyl groups into the thiazolidine ring by an expandase, deacetoxycephalosporin C synthase, DAOCS (the *cefE* gene product). This is then converted to deacetylcephalosporin C by DACS, deacetylcephalosporin C synthase, encoded by the *cefF* gene which hydroxylates the remaining C-3 methyl group. Deacetylcephalosporin C is then converted to cephamycin C by modification of the C-3 hydroxymethyl group and an addition of a methoxy group at C-7 (Jensen and Demain, 1995). These last three steps in the pathway are catalyzed by an o-carbamoyl transferase, a 7 α-hydroxylase and a methyl transferase activity presumably encoded by three genes, *cmcH, I* and *J* . The genes encoding all of these biosynthetic enzymes are clustered in 35 kb of DNA (Aharonowitz and Cohen, 1992; Perez-Llarena and Liras *et al.*, 1997; Alexander, 1998). The more recent additions to this gene cluster were the determination of the location of *cmcI* and *cmcJ* through sequencing by D. Alexander (1998) and the discovery of three additional open reading frames between the *cmcH* and the *lat* gene by

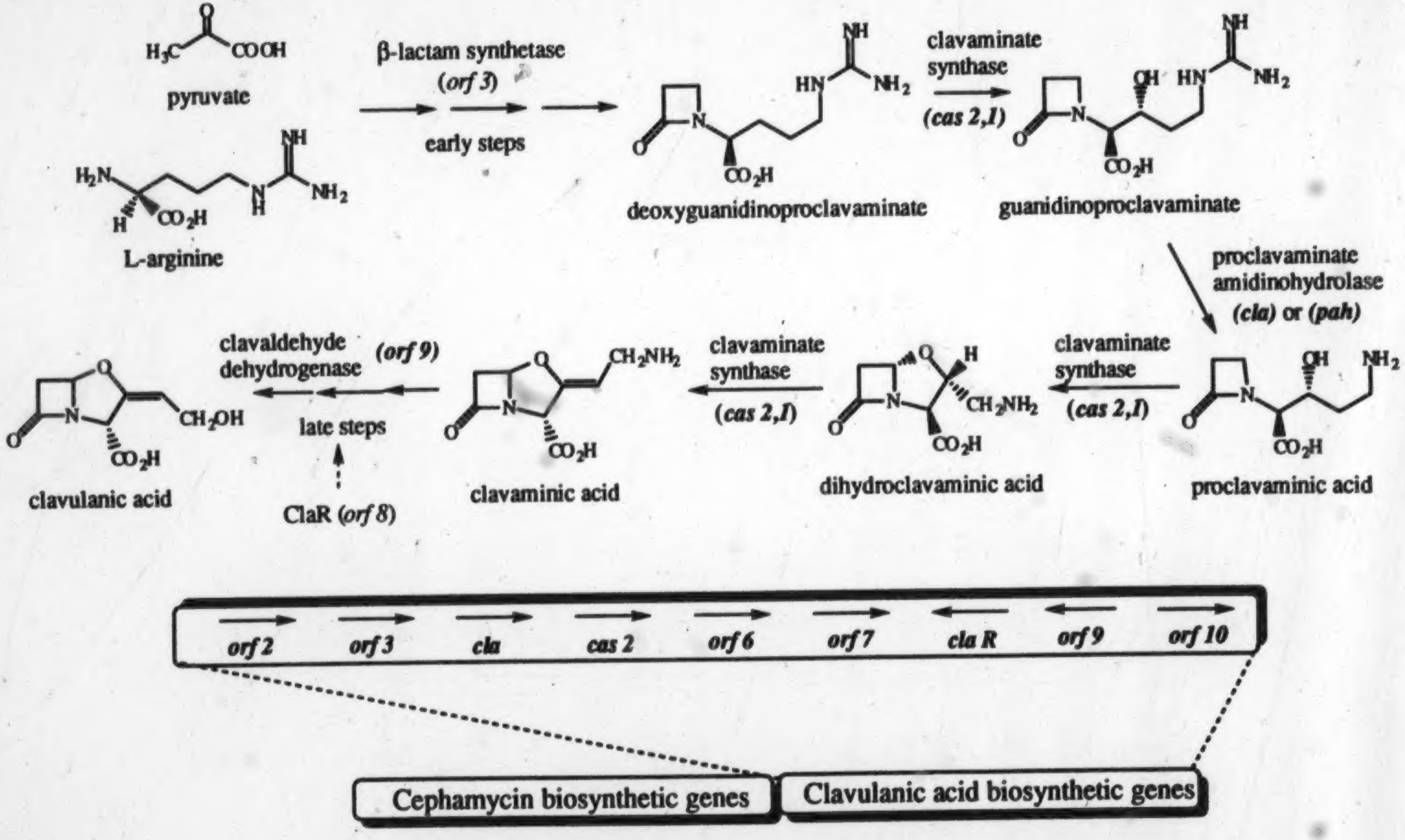
Figure 5. Cephamycin C biosynthetic pathway. General pathway for the synthesis of penicillin N and cephamycin C in *Streptomyces clavuligerus*. The enzymes and their corresponding genes are shown at each step. The Cephamycin C biosynthetic gene cluster is adjacent to the clavulanic acid gene cluster. The respective location of each gene involved is shown.


Lysine
lysine ε-amino transferase
α-amino adipate
cysteine
valine
ACVS
(pcb AB)
ACV
IPNS/cyclase
(pcb C)
Isopenicillin N
IPNE/epimerase
(cef D)
Penicillin N
DAOCS/ expandase
(cef E)
Deacetoxycephalosporin C
DACS (cef F)
Deacetylcephalosporin C
acetyltransferase
(cef G)
Cephalosporin C
O-carbamoyl transferase
(cmc H)
7 α hydrolase (cmc I)
methyl transferase (cmc J)
Cephamycin C
—OCH₃
CH₂OH
CH₃
bla
pbp A
cmc T
pcd
cef E
cef D
cmc I
cmc J
cef F
cmc H
cca R
orf 11
blp
lat
pcb AB
pcb C
pcb R
Cephamycin biosynthetic genes
Clavulanic acid biosynthetic genes

Perez-Llarena and Liras *et al.* (1997). Of the ORFs reported by Perez-Llarena and Liras *et al.*, the first of the three ORFs which is just downstream of *cmcH* codes for a transcriptional regulator important for regulation of expression of the cephamycin, clavulanic acid and clavam biosynthetic genes. Disruption of the *ccaR* gene, cephamycin, clavulanic acid regulator, resulted in elimination of both cephamycin and clavulanic acid production (Perez-Llarena and Liras *et al.*, 1997). Clavam production in a *ccaR* mutant is also abolished (Jensen, personal communication). The second gene, *orf11*, that was discovered downstream of *ccaR* does not resemble any known genes. The third gene, *blp*, lies between *orf11* and *lat* and it resembles the *bli* gene which encodes the β-lactamase inhibitory protein, BLIP. The *blp* gene codes for a protein designated BLP, BLIP-like protein, of 20.29 kDa containing a 28 amino acid N-terminal signal peptide. The presence of *blp* in the cephamycin cluster is interesting from the perspective of determination of the location of the *bli* gene and from this in attempting to infer the functions of BLP and BLIP. The genes encoding cephamycin C biosynthetic enzymes are clustered next to the genes encoding enzymes for clavulanic acid biosynthesis.

Current knowledge of the clavulanic acid biosynthetic pathway and the location of the genes involved is shown in Figure 6. The primary metabolites of clavulanic acid are pyruvate and L-arginine. The monocyclic deoxyguanidinoproclavaminate is formed through the initial condensation of pyruvate and L-arginine followed by some early steps, one of which involves the formation of the β-lactam ring by the product of the *orf3* gene, β-lactam synthetase (Bachmann *et al.*, 1998). The intermediate, deoxyguanidinoproclavaminate, is then hydroxylated by the product of the *cas2* gene, which is CAS2, clavaminate synthase. This is followed by removal of the guanidino group from the side chain of the arginine component by the product of the *cla* gene which is proclavaminate amidinohydrolase to generate proclavaminic acid. Proclavaminic acid is then cyclized and oxidized to dihydroclavaminic acid, the first bicyclic intermediate in the pathway, by clavaminate synthase encoded by the *cas2* gene. The next step which is the desaturation of the C-2-side chain carbon bond to generate clavaminic acid is also catalysed by clavaminate synthase. Clavaminic acid is then converted to clavulanic acid by late reactions believed to be catalysed by the products of *orf7*, *orf9* and *orf10* since the transcripts of these genes are absent in a *claR* mutant blocked in clavulanic acid production (Paradkar *et al.*, 1998). It is believed that the *claR* gene product is a transcriptional regulator that regulates the expression of the late

Figure 6. Clavulanic acid biosynthetic pathway. The clavulanic acid biosynthetic pathway in *Streptomyces clavuligerus* is shown. The metabolic precursors are pyruvate and L-arginine. The known intermediates and the enzymes that have been characterized are included in the pathway. The genes encoding clavulanic acid biosynthetic enzymes are clustered and are located adjacent to the cephamycin C biosynthetic genes.


pyruvate
L-arginine
β-lactam synthetase
(orf 3)
early steps
deoxyguanidinoproclavaminate
clavaminate
synthase
(cas 2,1)
guanidinoproclavaminate
proclavaminate
amidinohydrolase
(cla) or (pah)
proclavaminic acid
clavaminate
synthase
(cas 2,1)
dihydroclavaminic acid
clavaminate
synthase
(cas 2,1)
clavaminic acid
clavaldehyde
dehydrogenase
(orf 9)
late steps
ClaR (orf 8)
clavulanic acid
orf 2
orf 3
cla
cas 2
orf 6
orf 7
cla R
orf 9
orf 10
Cephamycin biosynthetic genes
Clavulanic acid biosynthetic genes

genes involved in converting clavaminic acid to clavulanic acid as disruption of the *claR* gene results in accumulation of clavaminic acid (Paradkar *et al*., 1998). The enzymes which catalyse late reactions have not yet been isolated except for clavaldehyde dehydrogenase which catalyzes the final reduction of clavaldehyde to clavulanic acid.

Nine additional ORFs further downstream of *orf10* which are not shown in the figure have been sequenced however their role in clavulanic acid biosynthesis is unknown (C. Anders, R. H. Mosher and S. E. Jensen, unpublished results). To date, the complete clavulanic acid biosynthetic pathway is believed to begin with *orf2* and extends to *orf10* which encompasses a 12 kb region of DNA. Although only *cla, cas2* and *claR* have been well characterized, gene disruption mutants have been created in all of *orfs 2 -10*. These mutants confirmed the involvement of these gene products in clavulanic acid production (K. Elder, 1998; A. Wong, K. Aidoo, A. Paradkar and S. E. Jensen, unpublished results) however the exact biochemistry of the reactions they catalyse is unclear.

The case of the clavaminate synthase gene, *cas2*, is interesting in that a paralog gene, *cas1*, which codes for a second clavaminate synthase isoenzyme, exists in a region of the *S. clavuligerus* genome that is at least 20 kbs away from *cas2*. The *cas1* and *cas2* genes have 87 % identity and the corresponding proteins show 82 % identity at the amino acid sequence level (Marsh *et al*., 1992). *cas1* is able to complement a disrupted form of the *cas2* gene and therefore allows for clavulanic acid production in a *cas2* mutant under conditions in which *cas1* is expressed. When Paradkar and Jensen (1995) disrupted the *cas2* gene, they found that although clavulanic acid production was blocked in starch-asparagine minimun medium (SA), in soy fermentation medium, clavulanic acid was produced. Since only the transcript for *cas2* is seen in wild type cultures grown in SA medium while both the cas2 and *cas1* genes are expressed in soy medium, the clavulanic acid detected in the *cas2* mutant grown in soy suggests that *cas1* is able to complement the disrupted *cas2* gene.

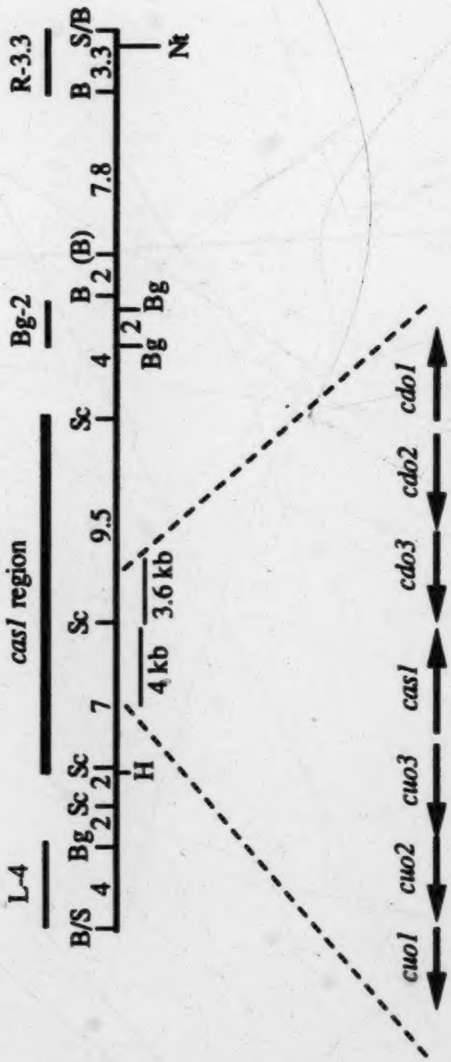
The *cas2* gene, located in the clavulanic acid biosynthetic gene cluster, is adjacent to the cephamycin biosynthetic cluster. The homologous *cas1* gene is located in a cluster of genes believed to be involved in biosynthesis of the antipodal clavam metabolites of *S. clavuligerus* as determined from gene disruption experiments (Mosher *et al*., 1998). The location of this cluster with respect to the cephamycin/clavulanic acid biosynthetic cluster is known only to be greater than 20 kilobases away (Marsh *et al*., 1992).

Little is known about the biochemistry or enzymes involved in clavam biosynthesis except that clavams and clavulanic acid biosynthesis share a common pathway until the clavaminic acid branch point (Egan *et al.*, 1997). Clavaminic acid, formed from the initial condensation of pyruvate and L-arginine followed by a series of reactions involving amidinohydrolase and CAS2/1 as outlined in Figure 6, has been shown to be a precursor of both clavulanic acid and the clavams by feeding studies using radioactive substrates. The products of the *cas1* and *cas2* genes both contribute to the clavaminic acid pool available for clavam and clavulanic acid biosynthesis. The *cas1* gene differs from the *cas2* gene in its expression pattern. *cas1* is a clavam biosynthetic gene in that it is not expressed in SA medium where all other clavam biosynthetic genes are also not expressed and hence clavams are not produced (Paradkar and Jensen, 1995). Disruption of *cas1* resulted in a significant decrease in clavam production in soy medium grown cultures (Mosher *et al.*, 1998). The small amounts of clavams produced are derived from the common clavaminic acid intermediate of the clavulanic acid/clavam biosynthetic pathway produced by the CAS2 enzyme which is also expressed in soy grown cultures. Subsequent steps involved in the conversion of clavaminic acid to the individual clavams beyond the clavaminic acid branch point have been hypothesized however no experimental data exists to support the reactions proposed (Janc *et al.*, 1993).

Figure 7 shows what is known of the region in the vicinity of the *cas1* gene. The restriction map is that determined for the insert in cosmid p53 (A. Wong, A. Paradkar and S. E. Jensen, unpublished), a stable derivative of NL1D1, a cosmid obtained from a genomic library of *S. clavuligerus* DNA by Southern hybridization using an oligonucleotide probe based on the *cas1* sequence (Mosher *et al.*, unpublished). Three ORF*s* have been identified upstream of the *cas1* gene. *cuo1*, *cuo2* and *cuo3* , *cas1* upstream ORFs, are oriented divergently to *cas1*. The *cuo1* gene product is known to be involved in clavam production as disruption of the *cuo1* gene resulted in a mutant that produces no detectable levels of clavam-2-carboxylate or hydroxymethylclavam. However, clavulanic acid was detected and wild type levels of cephamycin C were produced. Sequence homology searches revealed that *cuo1* shows greater than 40 % identity with aldo/keto reductases and dehydrogenases. *cuo2* and *cuo3* resemble no known proteins in the literature and have not been studied further (Mosher *et al.*, 1998).

Three ORFs have also been identified in the region downstream of the *cas1* gene. *cdo1* and *cdo2* , *cas1* downstream ORFs, are oriented in the opposite direction from the

Figure 7. Clavam biosynthetic gene cluster. Location and orientation of genes involved in clavam biosynthesis with respect to the *cas1* gene in cosmid p53 is shown. ORFs downstream of the *cas1* gene are designated *cdo*, *c**cas1* downstream orf, while those located upstream of the *cas1* gene are *cuo*, *c**as1* upstream orf. Abbreviations for the restrictions sites are as follows: B, *Bam* HI; Bg, *Bgl* II; H, *Hind* III; Nt, *Not* I; S, *Sal* I; Sc, *Sac* I. The heavy lines mark the *cas1* region, the left (L-4) and right (R-3.3) end regions of the cloned fragment containing the *cas1* area and an internal fragment (Bgl-1.6) that will be shown to be linked to the *bli* gene.


L-4
cas1 region
Bg-2
R-3.3
B/S
4
Bg
2
Sc
2
Sc
7
Sc
9.5
Sc
4
2
B
2
(B)
7.8
B
3.3
S/B
H
4 kb
3.6 kb
Bg
Bg
Nt
cuo1
cuo2
cuo3
cas1
cdo3
cdo2
cdo1

cas1 gene while *cdo3* is oriented in the same direction as *cas1*. The amino acid sequence of CDO1 shows 25% identity to the *cefG* gene product which is the deacetylcephalosporin C acetyltransferase enzyme of the Cephalosporin C biosynthetic pathway. The *cdo2* gene product shows ca. 31% identity to pyridoxal phosphate-dependent aminotransferases from *B. subtilis* and *E. coli* and 32% identity with pyridoxal phosphate-dependent dialkylglycine decarboxylase from *Pseudomonas cepacia*. The involvement of the *cdo1* and *cdo2* gene products in clavam biosynthesis is evident in a *cdo1/cdo2* deletion mutant which lacks part of both the *cdo1* and *cdo2* genes and therefore is presumed to be defective in production of both of these gene products. The *cdo1/cdo2* mutant, like the *cuo1* mutant, does not produce clavam-2-carboxylate, clavaminic acid or hydroxymethylclavam. Clavulanic acid and cephamycin C production, however, are unaffected (Mosher *et al.*, 1998).

Three interesting similarities exist between the cephamycin/clavulanic acid biosynthetic gene cluster and the clavam biosynthetic gene cluster. The first is the existence of the isoenzymes: CAS2, encoded in the clavulanic acid cluster (Aidoo *et al.*, 1994), and CAS1, encoded in the clavam cluster (Mosher *et al.*, 1998). The second is the finding that the first ORF upstream of the *cas1* gene which encodes CDO1 shows similarity to the *cefG* gene product in the Cephalosporin C biosynthetic pathway (Mosher *et al.*, 1998). And third, the presence of the *blp* gene, in the cephamycin C/clavulanic acid gene cluster (Perez-Llarena and Liras *et al.*, 1997) is interesting in that it raises the possibility that like *cas2* /*cas1* and the *cdo1* and *cefG* pair, perhaps the *bli* gene is near this second cluster which contains clavam biosynthetic genes.

Marsh *et al.* (1992) proposed that the isoenzymes CAS1 and CAS2 arose by gene duplication. The occurrence of gene duplication/amplification is a common phenomenon in bacteria and is determined to take place at a frequency of 10^{-1} to 10^{-5} (Stark and Wahl, 1984). Gene duplication in response to selective pressure was documented by Rigby *et al.* in 1974. They showed that catabolic genes such as the ribitol dehydrogenase gene of *Klebsiella aerogenes* which is required for the culture to grown on xylitol, a poorly metabolizable carbon source, is amplified to compensate for the low substrate specificity of the dehydrogenase for xylitol, by successive generations during growth in the presence of xylitol alone (Rigby *et al.*, 1974). Their study showed that the improved ability to grow in xylitol in successive generations by amplification of the relevant gene occurred by "spontaneous evolution" as well as in response to specific mutagens. He proposed that gene duplication in response to selective pressure was the necessary first step in order for divergent evolution to eventually generate gene families or isoenzymes. Furthermore, changes in enzyme specificity require the accumulation of multiple mutations over time which can only take place in a duplicated silent gene without compromising the organism's ability to grow. The presence of mutations may interfere with proper folding of the protein resulting in a silent gene that becomes heterogeneous within a population. However, over time, through the occurrence of a compensating mutation or a reversion mutation and the process of natural selection, proper folding will then give rise to a new enzyme family or an isoenzyme. Rigby *et al*. (1974) cite the example of serine proteases, trypsin and chymotrypsin enzymes as evidence in support of this hypothesis. Although 80 % of the surface residues of these proteins are different, their tertiary structures are almost superimposable. As proper folding determines the ability of a silent gene to become 'useful', they propose that the tertiary structure of the enzyme determines its evolutionary potential in that the more stringent the requirement of a certain folding structure for activity, the lower the frequency of attaining that specific tertiary structure via multiple mutations.

The phenomena of gene amplification and instability are well known in *Streptomyces* genetics. Instability in the genome can give rise to spontaneous deletion events that occur at a frequency of 0.1 to 1 % as indicated by the loss of particular phenotypes such as antibiotic resistance among progeny in spore plating (Hopwood and Kieser, 1990) and loss of the A-factor gene *afsA* in *S. bikiniensis* and *S. griseus* or the agarase gene in *S. coelicolor* A3(2) (Hutter and Eckhardt, 1988). With genetic rearrangements that lead to deletions are those which lead to amplification of DNA sequences. Certain amplification events may lead to an increase in resistance to antibiotics such as spectinomycin in *S. achromogenes* subsp. *rubradirus* (Hornemann *et al*., 1987) and kanamycin in *S. rimosus* (Potekhin and Danilenko, 1985) while others have no detectable phenotypic trait such as the amplification of large nonessential regions in *S. glaucescens* (Hopwood and Kieser, 1990).

If the *bli* and *blp* genes arose by gene duplication as presumably did *cas1*/*cas2* and perhaps *cdo1* and *cefG* as well, then the presence of the *blp* gene in the cephamycin C/clavulanic acid cluster would warrant a search for the *bli* gene in the vicinity of the clavam cluster. Furthermore, should *bli* be linked to clavam genes, then this would imply a common functional context for BLIP and the clavam metabolites of *S. clavuligerus*, as genes encoding products involved in secondary metabolite production and resistance are invariably clustered. Some examples of clustering of antibiotic biosynthetic genes include the clavam biosynthetic genes in *S. clavuligerus* (Mosher *et al.*, 1998); cephamycin and clavulanic acid biosynthetic genes in *S. clavuligerus*, *Streptomyces jumonjinensis* and *Streptomyces katsurahamanus* (Ward and Hodgson, 1993); the macrolide antibiotic carbomycin from *Streptomyces thermotolerans* (Epp *et al.*, 1989); streptomycin in *Streptomyces griseus* (Mansouri *et al.*, 1989); and many of the polyketide biosynthetic and resistance genes of *S. coelicolor*, *S. glaucescens*, *S. peucetius*, *S. fradiae* as summarized by Hopwood and Sherman (1990).

Recent approaches to discovery and determination of the function of new genes involves sequencing, homology comparison with a bank of existing genes and disruption of the coding region of the gene of interest by the introduction of an assayable marker, generally an antibiotic resistant marker followed by characterization of the mutant. Although the use of gene disruption for function determination in *Streptomyces* has been reported in the literature prior to 1990, gene disruption in *S. clavuligerus* was first done by Aidoo *et al.* (1994) to show that the *cla* gene just upstream of the *cas2* gene in the clavulanic acid gene cluster was essential for clavulanic acid production under some nutritonal conditions. The *cla* gene encodes an amidinohydrolase, an enzyme that was subsequently shown to convert guanidinoproclavaminate to proclavaminate in the clavulanic acid biosynthetic pathway (Wu *et al.*, 1995). Since then gene disruption has been used extensively in *S. clavuligerus* for determination of the function of newly discovered genes (Mosher *et al.*, 1998; Paradkar *et al.*, 1998; Perez-Llarena and Liras *et al.*, 1997; Perez-Llarena and Martin *et al.*, 1997). Although the principle of gene disruption is essentially the replacement of a wild type chromosomal copy of the gene of interest with a mutated cloned copy of the same gene by homologous gene replacement, the procedure involved in the disruption of a gene in *Streptomyces* includes additional steps due to the filamentous nature and the multinucleiodal vegetative state of *Streptomyces*. Although they are Gram positive bacteria, *Streptomyces* are more like fungi in that they grow in the form of branched networks of filamentous mycelia in surface and liquid cultures. Like fungi, their mycelia grow by linear apical extension. In *Streptomyces hygroscopicus* hyphal elongation has been shown to occur in the first 20 µm and exponential branching in the following 100 µm of the length of the hyphae (Miguelez *et al.*, 1992). Although cross walls exist in the filaments, histological staining of nuclear materials has shown that the size and shape of nucleoids vary greatly along the length of the mycelia. This heterogeneity is believed to be a result of the increase in DNA synthesis towards the tip of the hyphae as well as the incomplete segregation of the chromosomes after replication; this latter event being correlated with the expansion of hyphal walls during elongation. On surface media, the growth of *Streptomyces* occurs in the form of a subsurface mat of vegetative mycelia follow by differentiation into aerial mycelia which then give rise to uninucleate spores. The multinucleiodal state of *Streptomyces* mycelia exists throughout their life cycle except in the uninucleate spores. For this reason, in gene replacement experiments, plasmids containing the gene of interest usually disrupted by a selectable antibiotic marker, are introduced into lysozyme generated protoplasts. Following transformation by plasmid DNA, protoplasts are regenerated for 16 to 24 hours after which antibiotic selection is applied by agar overlay. Transformants are then regenerated on solid media, taken through the multinucleate substrate mycelial and aerial mycelial phases and then sporulated to induce the uninucleoidal state. Antibiotic resistant colonies are then plated on non selective agar media and allowed to sporulate. During subsequent growth as vegetative and aerial mycelia and then sporulation, exogenous plasmid is lost unless stably integrated in the genome by homologous single or double crossovers. Crossover frequency is generally dependent on the length of homologous sequences however, the frequency of single crossovers is greater than that of double crossovers. The individual spores obtained from non-selective growth can be screened for those containing the disrupted gene by replica plating. As the disruption plasmid contains two different antibiotic resistant markers, one in the disrupted gene of interest and the second in the plasmid vector, individual uninucleate spores containing an integrated copy of the disrupted gene can be identified by the loss of the plasmid marker and the stable retention of the disruption marker.

A second peculiarity to gene disruption in *S. clavuligerus* specifically is the requirement that the plasmid to be transformed into *S. clavuligerus* must first be passaged through the intermediate host *Streptomyces lividans*. DNA from *E. coli* does not transform *S. clavuligerus* efficiently presumably due to *S. clavuligerus*' restriction systems (Bailey and Winstanley, 1986). Furthermore, even with the transformation of a plasmid isolated from *S. lividans*, *S. clavuligerus* protoplasts are first subjected to a 45 °C heat treatment to inhibit the restriction system in order to increase transformation frequency (Bailey and Winstanley, 1986).

Initial characterization of wild type strains or disrupted mutants with regard to their ability to produce antibiotics or other bioactive metabolites was done by the bioassay method whereby the growth or the inhibition of growth of an indicator organism is used as an indicator of the presence of a biologically active compound. Clavulanic acid was initially discovered by a bioassay technique designed to identify a compound that, in the presence of penicillin G, could inhibit the growth of a β-lactamase producing strain of *Klebsiella aeruginosa* (Baggaley *et al.*, 1997). Since then HPLC methods for the detection and quantitation of β-lactams involving the derivatization of the β-lactam with imidazole have been developed (Eckers *et al.*, 1996). The reaction of β-lactams such as clavulanic acid and the clavams with imidazole occurs at room temperature in 15 minutes (details are described in Section II.9.3.2), and the derivatized product is stable for at least one hour. In the derivatization reaction, the amide bond of the β-lactam ring is cleaved and a new C-N bond is formed between the carbonyl carbon and the nitrogen of imidazole. This cleavage of the β-lactam also triggers the opening of the oxazolidine ring structure. In the resulting β-lactam/imidazole adduct, the conjugation of the double bond in the imidazole moiety and the carbonyl group of the β-lactam compound results in a high absorption coefficient at 313 nm. The derivatization with imidazole also serves to improve the retention qualities in conventional reverse phase HPLC columns since clavulanic acid and similar β-lactam compounds such as the clavams are polar and not well retained otherwise. Derivatization with imidazole allows for chromatographic detection and separation of similar compounds such as clavulanic acid and the clavams such as clavam-2-carboxylate (Eckers *et al.*, 1996) and hydroxymethyl clavam (B. Barton, personal communication).

In this study, attempts to determine the function of the BLIP protein using the experimental approaches discussed above, are described. The gene replacement method was used to create a BLIP non producer and a BLIP/clavulanic acid double mutant. The mutants were then characterized with respect to the levels of the different β-lactams produced by HPLC, their resistance to Penicillin G by bioassays and their cell morphology by phase contrast microscopy. Genetic mapping of the *bli* gene was undertaken. Results of sequencing the region in the vicinity of the *bli* gene are discussed. And lastly, studies involving the nutritional regulation of the *bli* gene are reported.

II. MATERIALS AND METHODS

II.1 MATERIALS

II.1.1 Chemicals and antibiotics

The antibiotics thiostrepton and ampicillin were obtained from Sigma, St. Louis, MO. While hygromycin was obtained from Boehringer Mannheim, Laval, Quebec. [α-^{32}P]dCTP was obtained from Amersham, Arlington Heights, IL. The *Apa I/Bam* HI oligonucleotide linker and DNA sequencing primers were obtained from Dept. of Biological Sciences DNA Synthesis Laboratory, University of Alberta, Edmonton, Alberta. PCR primers WS4 and WS5, were obtained from W. S. Schroeder, Department of Biological Sciences, University of Alberta. Chemicals and reagents were purchased from Sigma. Reagents and buffers used in molecular biology research such as TRIS, EDTA, monobasic and dibasic sodium/potassium phosphates, NaOH, SDS, amino acids and the like were purchased from either ICN, Aurora, Ohio; Sigma, St. Louis, MO; BDH, Toronto, Ontario or Life Technologies, Gaithersburg, MD. Growth media were purchased from Difco Laboratories, Detroit, Michigan.

II.1.2 DNA modifying enzymes

Restriction endonucleases and DNA modifying enzymes were from Boehringer Mannheim, Indianapolis, IN; New England Biolabs, Beverly, MA; and Promega, Madison, WI. Taq polymerase for PCR was obtained from A. Hashimoto, Department of Biological Sciences, University of Alberta.

II.2 BACTERIAL STRAINS, PLASMIDS, COSMIDS AND CULTURE CONDITIONS AND GROWTH MEDIA

II.2.1 Bacterial strains

Streptomyces clavuligerus NRRL 3585 was obtained from the Northern Regional Research Laboratories, Peoria, Ill. *Streptomyces lividans* TK24 was obtained from T. Kieser, John Innes Institute, Norwich, England. The *claR* mutant was created by A. Paradkar by gene replacement (Paradkar *et al.*, 1998). The *ccaR* mutant was obtained from D. Alexander, Dept. of Biological Sciences, University of Alberta. *E. coli* MV1193 and DH5α were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. *E. coli* GM48 was obtained from L. Frost, Dept. of Biological Sciences, University of Alberta. *E. coli* ESS was obtained from A.L. Demain, Massachusetts Institute of Technology, Boston, MA.

II.2.2 Plasmids and cosmids

The cloning vectors used in this study were obtained from the following sources: pUC119 was a gift from J. Vieira, Waksman Institute of Microbiology, Rutgers University; pBR322 was obtained from ATCC; pIJ2925 (Bibb *et al.*, 1993) and pIJ702 (Katz *et al.*, 1983) were obtained from M.J. Bibb, John Innes Institute, Norwich, England; pJOE829 was obtained from J. Altenbuchner, University of Stuttgart, Stuttgart, Germany. pBlip2 was created by S.E. Jensen, Department of Biological Sciences, University of Alberta (unpublished results). NL1D1 was created by R.H. Mosher, N. Logsetti and S.E. Jensen (unpublished), University of Alberta. Cosmid p53 was isolated by A. Wong, A. Paradkar and S.E. Jensen, University of Alberta. pBIP was constructed by Doran *et al.* (1990). The plasmids and cosmids used in this work are described in Table 1.

II.2.3 Culture conditions and growth media

All *E. coli* strains were maintained as 20% glycerol stocks stored at -70 °C. For plasmid preparation, *E. coli* cultures were grown overnight at 37 °C in roller tubes in 2X YT [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl] containing ampicillin at 100 µg/ml. For generation and regeneration of competent cells, *E. coli* cells were grown and prepared as described in Chung *et al.* (1989).

All *Streptomyces* strains were maintained as 20% glycerol spore stocks at -70°C. For sporulation, *S. clavuligerus* was grown on ISP-3 medium (Difco Laboratories, Detroit, Michigan) for three weeks after which the spores were harvested as described in Hopwood *et al.* (1985). For sporulation of *Streptomyces lividans*, R2YE (see next section for recipe) without sucrose was used as a growth medium. For genomic and plasmid DNA isolation from *Streptomyces*, the cultures were grown in trypticase soy broth (TSB) and 1% starch (TSBS) with shaking at 280 rpm for 2 to 3 days until a thick culture was obtained. All *S. clavuligerus* cultures were incubated at 28 °C while *S. lividans* cultures were incubated at 30 °C in flasks equipped with wire springs. *S. lividans* carrying pJOE829 was grown in TSBS containing 50 µg/ml of hygromycin while pIJ702 containing strains were grown in TSBS containing 50 µg/ml of

Table I. Plasmid and cosmid vectors used in this study.

	PLASMID/COSMID	INSERT	RESISTANCE MARKER	SOURCE
pIJ2925	pUC based plasmid containing *Bgl* II sites flanking multiple cloning region	N/A	ampicillin	Bibb *et al.*, 1993
pJOE829	*Streptomyces* cloning vector	N/A	hygromycin	J. Altenbuchner, University of Stuttgart, Stuttgart, Germany
pIJ702	*Streptomyces* cloning vector, source of 1 kB *tsr* gene	N/A	thiostrepton	Katz *et al.*, 1983
pLAFR3	Broad host range cosmid vector	No insert; vector: 22 kilobases	tetracycline	Staskawicz *et al.*, 1987
pFD666	*Streptomyces* cloning vector	No insert; vector: 5.25 kilobases	neomycin	Denis *et al*, 1992
pBIP	pLAFR3 based cosmid containing 13.5 kB*Streptomyces* fragment	13.5 kilobase fragment containing *bli* gene and sequences extending 4 kB left and 9 kB right of *bli* gene	tetracycline	Doran *et al.*, 1990
pBLIP2	pUC119 plasmid containing *bli* gene	*bli* gene on an 840 bp *Cla* I/*Kpn* I fragment	ampicillin	S.E. Jensen, University of Alberta, Edmonton, Alberta
NL1D1	pFD666 based cosmid, isolated by Southern hybridization of DNA library with oligonucleotide complementary to *cas1* gene	> 45 kB insert containing clavam biosynthetic genes	neomycin	R.H. Mosher, N. Logsetti and S.E. Jensen, University of Albert
cosmid p53	stable derivative of NL1D1	> 45 kB insert containing clavam biosynthetic genes	neomycin	A. Wong, A. Paradkar and S.E. Jensen, University of Alberta

thiostrepton. *S. clavuligerus* strains containing pIJ702 or pJOE829 were grown in TSBS containing 5 μg/ml of thiostrepton or 250 μg/ml of hygromycin respectively.

II.2.3.1 Culture conditions and media used in *Streptomyces* protoplast generation and regeneration

For protoplasting, *S. lividans* TK24 was grown in YEME [0.3% Difco yeast extract (w/v), 0.5% Difco Bacto-peptone (w/v), 0.3% malt extract (w/v), 1% glucose (w/v), 34% sucrose (w/v), 0.005 M $MgCl_2$ and 0.5% glycine (w/v)] as described in Hopwood *et al.* (1985). (All values will be given as w/v unless otherwise indicated.) For generating *S. clavuligerus* protoplasts, the culture was grown according to a method obtained from researchers at Smithkline Beecham. A 25 ml seed culture of TSB and 1% maltose (TSBM) was inoculated with a spore inoculum and grown for 3 days at 25 °C in a spring flask. A 25 ml TSBM/YEMEM combination medium [10 ml TSB, 15 ml YEME, 1% maltose, and 0.005 M $MgCl_2$] was inoculated with 1 ml of the TSBM seed and incubated with shaking in a spring flask at 25 °C for 18 hours. *S. lividans* protoplasts were regenerated in R2YE [0.3 M sucrose, 1.4 mM K_2SO_4, 47 mM $MgCl_2\cdot 6H_2O$, 1% glucose, 0.01% Difco casamino acids, 0.5% Difco yeast extract, 25 mM TES, 147 μM KH_2PO_4, 8 mM $CaCl_2\cdot 6H_2O$, 10.4 mM L-proline, 0.2% trace elements (v/v) and 2.2% agar, pH 7.0; trace elements are added after autoclaving]. Trace elements for R2YE included 0.004% $ZnCl_2$, 0.02% $FeCl_3\cdot 6H_2O$, 0.001% $CuCl_2\cdot 2H_2O$, 0.001% $MnCl_2\cdot 4H_2O$, 0.001% $Na_2B_4O_7\cdot 10\,H_2O$ and 0.001% $(NH_4)_6 Mo_7O_{24}\cdot 4H_2O$. *S. clavuligerus* protoplasts were regenerated on R5B medium (Bailey and Winstanley, 1986) which contained 0.3 M sucrose, 65 mM sodium glutamate, 24 mM $MgCl_2\cdot 6H_2O$, 0.1% casamino acids, 0.20 mM $MgSO_4\cdot 7H_2O$, 1% starch, 25 mM TES, 37 mM KH_2PO_4, 25 mM $CaCl_2\cdot 2H_2O$, 0.2% R2YE trace elements (v/v) and 2.2% agar, pH 7.2. For selection of *Streptomyces* transformants, plates containing regenerated protoplasts were overlayed with 2.5 ml of soft nutrient agar (SNA) [0.8% Difco nutrient broth and 0.3% agar] containing thiostrepton at 375 μg/ml for *S. lividans* and 50 μg/ml for *S. clavuligerus* to give a final concentration of 37.5 μg/ml of thiostrepton in *S. lividans* and 5 μg/ml in *S. clavuligerus*. Replica plating of *S. clavuligerus* isolated colonies was done on MYM [0.4% maltose, 0.4% yeast extract, 1% malt extract, 1.5% agar] containing 10 μg/ml of thiostrepton and MYM containing 250 μg/ml of hygromycin.

II.2.3.2 Media used in nutritional studies.

All values are given as percent w/v unless otherwise indicated. Starch asparagine (SA) medium (Aharonowitz, 1978) contained 1% starch, 0.2% L-asparagine, 2.1% MOPS, 0.44% K_2HPO_4, 0.06% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 6.8 and 1 ml of SA trace elements was added per liter of medium after autoclaving. SA trace elements contains 1.3 mg/ml $CaCl_2 \cdot 3H_2O$, 1 mg/ml $FeSO_4 \cdot 7H_2O$, 1 mg/ml $MnCl_2 \cdot 4H_2O$, and 1 mg/ml $ZnSO_4 \cdot 7H_2O$. Glycerol-sucrose-proline-glutamate medium, GSPG, (Romero *et al.*, 1986) contained 1.5% glycerol, 2.0% sucrose, 0.25% L-proline, 0.15% L-glutamic acid, 0.5% NaCl, 0.2% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0 and filter sterilized GSPG trace elements was added after autoclaving to a final concentration of 10% (v/v). GSPG trace elements contains 4 mg/ml $CaCl_2$, 1 mg/ml $MnCl_2 \cdot 4H_2O$, 1 mg/ml $FeCl_3 \cdot 6H_2O$ and 0.5 mg/ml $ZnCl_2$. Soy fermentation medium (Salowe *et al.*, 1990) contained 0.15% soybean flour, 0.47% starch, and 0.001% KH_2PO_4. The pH was adjusted to 6.8 and 2 ml of filter sterilized $FeSO_4 \cdot 7H_2O$, 10 mg/ml, was added after autoclaving.

II.3 *E. COLI* AND *STREPTOMYCES* DNA ISOLATION

Plasmids and cosmids replicated in *E. coli* were isolated and purified according to the method of Birnboim and Doly (1979) as described in Sambrook *et al.* (1989). *Streptomyces* plasmids were isolated and purified by the alkaline lysis method of Kieser *et al.* (1984) as described in Hopwood *et al.* (1985). Genomic DNA from *Streptomyces* was purified according the method of S.H. Fisher as described in Hopwood *et al.* (1985). DNA fragments from restriction digests, separated by agarose gel electrophoresis, were isolated and purified as described in Zhen and Swank (1993) with the following modifications. The DNA fragments were separated by electrophoresis on agarose gels which did not contain ethidium bromide. The gels were then stained in ethidium bromide staining solution containing 1 mM EDTA, pH 8.0, and destained in distilled H_2O containing 1 mM EDTA, pH 8.0, for visualization of DNA fragments.

II.4 QUANTITATION OF TOTAL DNA FROM *STREPTOMYCES* CULTURES AS A MEASURE OF GROWTH.

The method used for measuring growth of *Streptomyces* cultures by quantitation of the DNA content was based on a method obtained from W. H. Holms, BioFlux Ltd., Robertson Institute of Biotechnology, Glasgow, Scotland. The procedure with slight modifications was as follows: cultures, grown to the respective age, were harvested by centrifugation at 27 100 x g for 15 minutes. The mycelial pellet from each 10-15 ml culture sample was resuspended in an equal volume of cold 0.2 M perchloric acid (PCA) and kept at 4 °C for at least 16 hours or until all samples in the time course experiment had been harvested. The mycelial suspension was then centrifuged a second time and the pellet resuspended in 10-15 ml of 0.5 M PCA. This mycelial suspension in 0.5 M PCA was then incubated at 70 °C for 2 hours and subjected to centrifugation at 27 000 x g at room temperature. The supernatant obtained by centrifugation was then assayed for DNA content by a scaled down version of the Burton method (Burton, 1956) which was as follows: one milliter of Burton reagent, made as described in Daniels *et al.*, (1994), was reacted with 0.5 ml of the sample (0.5 M PCA soluble fraction) at room temperature for 16-24 hours. The Burton reagent contained 1.5% diphenylamine crystals (w/v) in acetic acid, 1.5% of reagent-grade concentrated sulfuric acid and 0.5% of a 16 mg/ml aqueous acetaldehyde solution (1 ml in 50 ml of distilled H_2O). The aqueous acetaldehyde component of the Burton reagent was added on the day of use. The absorbance at 600 nm was measured and amounts of DNA were determined using a standard curve of salmon sperm DNA. Half milliliter amounts of DNA standards, prepared at concentrations of 0.02-0.2 mg/ml in 0.5 M PCA were reacted with 1 ml of Burton reagent and incubated as the test samples. The blank consisted of 0.5 ml of 0.5 M PCA reacted with 1 ml of the Burton reagent.

II.5 GENERATION OF *E. COLI* COMPETENT CELLS AND TRANSFORMATION

E. coli cells were made competent by the TSS method (Chung *et al.* 1989). An overnight culture was used to inoculate 50 ml of LB broth at 1% (v/v). The culture was grown with shaking at 37 °C to an OD_{600} of 0.3-0.4. Cells were harvested by centrifugation for 10 minute at 3000 x g and resuspended to one-fifth its initial volume in 1X TSS. TSS, transformation and storage solution, was LB broth with 10% PEG8000, 5% (v/v) DMSO and 50 mM $MgCl_2 \cdot 6H_2O$. The pH of TSS was adjusted to 6.5 with HCl. DMSO was added after autoclaving. Competent cells were stored at -70

°C in 250 µl volumes. For transformation, the competent cells were first thawed on ice, the DNA was then added, and the DNA/competent cell mixture was incubated on ice for 30 minutes for DNA uptake. For recovery following the 30 minute DNA uptake step, 750 µl of LB broth was added to the competent cells and the suspension was incubated at 37 °C for 1 hour. The resulting culture was then plated on LB plates containing 100 µg/ml of ampicillin and incubated overnight at 37 °C.

II.6 *STREPTOMYCES* PROTOPLASTING AND TRANSFORMATION

S. lividans protoplasting, transformation and regeneration were as described in Hopwood *et al.* (1985) with the following modifications. Protoplasts, made and stored in 100 µl volumes, were thawed and then washed once with 500 µl of P buffer [Basal solution (0.3 M sucrose, 1.4 mM K_2SO_4, 9.4 mM $MgCl_2 \cdot 6H_2O$, 0.2% (v/v) R2YE trace elements) and 0.37 mM KH_2PO_4 solution, 25 mM $CaCl_2 \cdot 2H_2O$ solution and 25 mM TES (pH 7.2) solution]. They were then sedimented by centrifugation at 6500 rpm on a MicroCentaur benchtop centrifuge. All centrifugation steps for *Streptomyces* protoplast transformation were done at 6500 rpm in a MicroCentaur benchtop centrifuge. The pelleted protoplasts were resuspended in the last drop of buffer remaining in the tube after decanting. The ligation reaction mixture to be transformed (20 µl volume) was then added followed by the addition of 100 µl of T buffer (P buffer and 25% PEG1000), the solution was then mixed by pipetting up and down once and 1 ml of P buffer was then added immediately following mixing. The transformed protoplasts were then sedimented by centrifugation for 2 minutes at 6500 rpm. The pelleted protoplasts were then resuspended in 500 µl of new P buffer, plated on predried R2YE plates and incubated at 30 °C. When the plates showed a characteristically faint hazy appearance, usually within 24 hour of plating, they were overlayed with SNA containing 375 µg/ml of thiostrepton for an effective thiostrepton concentration of 37.5 µg/ml per plate.

The method used for *S. clavuligerus* protoplast formation, as described below, was based on the procedure outlined in Hopwood *et al.* (1985) with a few modifications by researchers at SmithKline Beecham. The *S. clavuligerus* culture grown as described in section II.2.3.1 was washed two times in 10.3% sucrose, pelleted by centrifugation at 3000 x g and frozen at -70 °C. Before protoplasting, the pellet was again washed with 10.3% sucrose. To generate protoplasts, the mycelial pellet was resuspended in 4 ml of a filtered sterilized (0.45 µm filtration cell,Costar) lysozyme solution [2 mg/ml of lysozyme (Sigma) in P buffer] and incubated at 25 °C for 15-60 minutes. The cell suspension was subjected to gentle inversion every 10 minutes. Protoplast formation was judged to be complete when no mycelial fragments were seen under phase contrast microscopy at 400 x magnification. The suspension was diluted with 5 ml of P buffer, mixed by inversion and filtered through a syringe containing sterile nonabsorbent cotton wool. The protoplasts were then sedimented by centrifugation at 1100 x g, washed once with P buffer and resuspended in P buffer at 0.25 times the original volume of the culture. The protoplasts were stored in 100 µl volumes at -70 °C by gradual freezing on ice at -70 °C. For transformation, the protoplasts were thawed, washed once with P buffer and pelleted by centrifugation at 6500 rpm for 2 minutes. The pellet was then resuspended in the drop of P buffer that remained after decanting and incubated in a water bath at 45 °C for 10 minutes. DNA was then added, the mixture was mixed by pipetting up and down once and 1 ml of T buffer was added. The suspension was centrifuged at 6500 rpm and the pellet was resuspended in 0.5 ml of P buffer. This was then plated on predried R5B plates. *S. clavuligerus* transformants were selected for by overlaying with 2.5 ml per plate of a thiostrepton/soft nutrient agar overlay (50 µg/ml of thiostrepton) for a final thiostrepton concentration of 5 µg/ml per plate.

II.7 CREATION OF MUTANTS

II.7.1 Disruption of the *bli* gene.

II.7.1.1 Insertion of an *Apa I/Bam* HI linker in *bli* gene.

An *Apa I/Bam* HI oligonucleotide linker was made having the sequence 5' GTGGATCCACGGCC 3'. Oligonucleotide concentration was determined as described in Sambrook *et al.* (1989). The oligonucleotide, dissolved in H_20, was annealed by incubation at 75 °C for 10 minutes and then cooled slowly to room temperature to produce the double stranded linker with 3' *Apa* I cohesive overhanging ends as shown below. The *Bam* HI recognition site is underlined.

```
     5'  GTGGATCCACGGCC  3'
3'  CCGGCACCTAGGTG  5'
```

pBLIP2 (see Table 1) was linearized by digestion with *Apa* I. The linearized plasmid was then extracted with phenol/chloroform, chloroform, and then precipitated with 98% ethanol. The DNA pellet was washed with 70% ethanol and redissolved in TE buffer.

The *Apa* I/*Bam* HI linker was ligated into the *Apa* I site midway in the *bli* gene using a 100 fold molar excess of linker (Sambrook *et al*., 1989) over plasmid DNA in a volume of 20 µl. *E. coli* MV1193 TSS competent cells were transformed with 20 µl of the ligation mixture, allowed to regenerate at 37 °C and then plated on LB +100 µg/ml of ampicillin. The positive clones (pBLIP3), were identified by restriction digest with *Bam* HI.

II.7.1.2 Disruption of the *bli* gene by *tsr* insertion

pBLIP3 was digested with *Bam* HI. The linearized plasmid was extracted with phenol/chloroform, chloroform, and precipitated with 98% ethanol. The DNA pellet was washed with 70% ethanol, and resuspended in TE buffer.

The thiostrepton resistant gene (*tsr*) was obtained by digesting the plasmid pIJ702 with *Bcl* I at 50 °C overnight. The restriction digest was separated on a 0.8% agarose gel and the 1.09 kb fragment containing the *tsr* gene was purified as described in section II.3.

The *tsr* gene was ligated into the linearized pBLIP3 using an insert to plasmid ratio of 5:1. The ligation mixture containing 20 ng of DNA was used to transform *E. coli* DH5α TSS competent cells as described in II.5. The resulting construct was called pUC*bli::tsr*.

II.7.1.3 Ligation of *bli::tsr* into pIJ2925

The pUC*bli::tsr* plasmid was digested with *Eco* RI and *Pst* I to removed the 1.9 kb *bli::tsr* fragment. This *bli::tsr* fragment was gel purified as described in section II.3 and ligated into the vector pIJ2925 similarly digested with *Eco* R1 and *Pst* I, using a 4:1 insert to plasmid ratio. From the ligation reaction, 20 ng of DNA was used to transform *E. coli* DH5α TSS competent cells. The positive clones, containing pIJ2925*bli::tsr*, were screened using the Xgal/IPTG blue/white method as described in Sambrook *et al*. (1989) and confirmed by digestion with *Bgl* II which should separate the 1.9 kb *bli::tsr* fragment from the plasmid.

II.7.2 Ligation of the *bli::tsr* fragment into pJOE829 and passage through *S. lividans*.

pIJ2925*bli::tsr* was digested with *Bgl* II to remove the *bli::tsr* insert from the plasmid. The *bli::tsr* fragment was separated on a 0.8% agarose gel, purified as described in section II.3, and then ligated into the *Bgl* II site of pJOE829 in an insert to plasmid ratio of 5:1 to generate pJOE*bli::tsr*. From the ligation reaction, 96 ng of DNA was used to transforme *S. lividans* protoplasts. Positive clones, *S. lividans* containing pJOE*bli::tsr*, were grown in TSB containing 50 μg/ml of thiostrepton and 50 μg/ml of hygromycin for plasmid isolation.

II.7.3 Transformation of *S. clavuligerus* and screening for mutants.

S. clavuligerus protoplasts were transformed with pJOE*bli::tsr* as described in section II.5. Thiostrepton resistant transformants from plates overlayed with 5 μg/ml of thiostrepton were patched onto MYM plates containing 10 μg/ml of thiostrepton and 250 μg/ml of hygromycin. Thiostrepton resistant and hygromycin resistant transformants (TsR/HygR) were plated on ISP-3 medium containing no antibiotic selection and allowed to sporulate. The spores were harvested, serially diluted in sterile distilled water and replated on ISP-3 medium to obtain isolated colonies, again without antibiotic selection. When sporulated, the isolated colonies were replica plated on MYM medium containing thiostrepton at 5 μg/ml and on MYM containing hygromycin at 250 μg/ml. Colonies that were thiostrepton resistant and hygromycin sensitive (TsR/HygS) were selected as the putative mutants.

II.8 CONFIRMATION OF GENE REPLACEMENT BY SOUTHERN HYBRIDIZATION.

II.8.1 Southern blotting

Genomic DNA, isolated as described in II.3, was digested with *Kpn* I and separated on a 0.8% agarose gel. The gel was subjected to acid depurination and blotted onto nylon membrane (Hybond-N) as described in Hopwood *et al.* (1985). The membrane was vacuum baked at 70 °C for 3 hours.

II.8.2 Hybridization

II.8.2.1 Probe labelling by nick translation

The 840 basepair *bli* probe was obtained by digesting pBLIP2 with *Cla* I and *Kpn* I, separated from plasmid DNA by agarose gel electrophoresis and purified as described in section II.3. The 1.09 kb *tsr* probe was obtained by digestion of pIJ702 with *Bcl* I and similarly purified. Twenty nanogram amounts of each DNA probe were labelled with α^{32}P-dCTP by nick translation according to the method in Hopwood *et al.*, (1985). The nick translation reaction was carried out at 15 °C for 2 hours. The reaction was stopped by the addition of 0.5 M EDTA to a final concentration of 0.125 M and the labelled probes were separated from unincorporated dNTPs by gel filtration through a G-25 column. The probes labelled by this procedure were denatured by incubation at 95 °C for 5 minutes followed by immediate cooling on ice.

II.8.2.2 Prehybridization, hybridization of blots and autoradiography.

Nylon membranes to be probed were prehybridized by incubation with salmon sperm DNA at a final concentration of 150 µg/ml in prehybridization solution [5 x SSPE (0.9 M NaCl, 0.05 M dibasic sodium phosphate, 5 mM EDTA pH 7.7), 5 x Denhardt's solution (0.1% (w/v) BSA fraction V, 0.1% (w/v) Ficoll, and 0.1% (w/v) polyvinylpyrrolidone) and 0.5% (w/v) SDS] for 4 hours at 65 °C. The labelled probes were then added and the blots were incubated at 65 °C overnight. The membranes were washed, as specified by the manufacturer (Amersham, Oakville, Ontario), twice with a solution of 2 x SSPE and 0.1% (w/v) SDS at room temperature for ten minutes each, then once with a solution of 1 x SSPE and 0.1% SDS at 65 °C for 15 minutes and a final high stringency wash of 0.1 x SSPE and 0.1% SDS at 65 °C for 15 minutes. The membranes were then exposed to X-ray film (Scientific Imaging Systems, Eastman Kodak Company, New Haven, N.Y.). Exposure occurred at -70 °C for 24 hours after which the films were developed in a film processor.

II.9 BLIP AND β-LACTAM ASSAYS

II.9.1 Culture conditions for BLIP and β-lactam production.

II.9.1.1 Method A: spore inoculum.

One hundred and twenty five milliliter Erlenmeyer flasks containing 25 ml of TSBS broth were inoculated with a glycerol spore suspension of *S. clavuligerus* and grown with shaking (280 rpm) at 28 °C. The cultures were harvested at 24 and 48 hours (exponential phase) and 72 and 96 hours (stationary phase) by centrifugation at 13 000 x g for 15 minutes. Culture supernatants were used for BLIP enzyme inhibition assays or antibiotic bioassays.

II.9.1.2 Method B: seed inoculum.

One hundred and twenty five milliliter Erlenmeyer flasks containing 25 ml of TSBS broth were inoculated with a spore suspension of *S. clavuligerus* and grown with shaking at 28 °C for 48 hours. This seed culture was then harvested by centrifugation at 13 000 x g for 15 minutes, washed two times with sterile distilled H_2O and resuspended in H_2O to give a suspension with an OD_{600} of 1. The resulting suspension was used as a 2-4 % (v/v) inoculum. In studies where wild type and mutants strains were being compared for β-lactam production, the TSBS seed cultures were inoculated with similar amounts of viable spores of each strain so that each seed culture would reach similar optical densities at harvest.

II.9.1.3 Clavulanic acid and clavam production.

The TSBS seed cultures were used to inoculate triplicate 25 ml amounts of soy media in 125 ml flasks to 2% (v/v). The cultures were grown for 48, 72 and 96 hours. The cultures were harvested by centrifugation at 13 000 x g and the culture supernatants were collected and assayed for β-lactams by C-18 reverse phase HPLC (see section II.9.3.2) or BLIP by western analysis.

II.9.1.4 BLIP production in various nutritional conditions.

The TSBS seed cultures described in section 8.1.2 were used to inoculate various media to 2-4% (v/v). The cultures were incubated with shaking until a thick cell suspension was obtained, usually 50-140 hours. The culture supernatants were harvested by centrifugation at 13 000 x g and assayed for BLIP by enzyme inhibition assay or western analysis.

II.9.2 BLIP assays.

II.9.2.1 Bactopenase inhibition assay.

The β-lactamase inhibition assay measured the rate of degradation of Penicillin G by the β-lactamase Bactopenase (Difco) in the presence or absence of inhibitor by spectrophotometrically following the absorbance of Penicillin G at 240 nm. Bactopenase, $2x10^4$ units, was preincubated at room temperature for 5 minutes with the sample to be assayed for β-lactamase inhibitory activity, final volume adjusted to 500 µl with 0.1 M phosphate buffer pH 7.0 . Following the five minute preincubation, the reaction was started with the addition of 500 µl of 0.3 mg/ml of Penicillin G (Sigma) in the above phosphate buffer and the decrease in absorbance at 240 nm was measured for five minutes at one minute intervals. (The standard β-lactamase inhibition assay therefore includes $2x10^4$ units of Bactopenase in a volume of 100 µl of 0.1 M phosphate buffer pH 7.0, 100 µl of sample, 300 µl of 0.1 M phosphate buffer pH 7, and 500 µl of 0.3 mg/mlPenicillin G solution.) In the uninhibited reaction, which is the degradation of Penicillin G in the absence of inhibitor, H_2O was preincubated with Bactopenase/buffer mix.

Culture supernatants to be asayed for Bactopenase inhibition were prepared with or without heat inactivation. Heat inactivation involved incubation at 95 °C for 30 minutes followed by centrifugation at 13 000 rpm on a MicroCentaur bench top centrifuge. Samples were then assayed for β-lactamase inhibition and the rates so obtained were compared to those of the same samples that had not been heat inactivated. Rates obtained for culture supernatants assayed without heat inactivation represented the sum of β-lactamase inhibitory activity attributable to both clavulanic acid and BLIP. Rates determined for samples that had been heat treated measured the inhibitory activity due to clavulanic acid alone. The difference between the heat inactivated and the untreated samples was a measure of the amount of BLIP present.

BLIP activity could also be removed from culture supernatants by methanol precipitation. The samples were diluted with an equal volume of methanol, incubated on ice for 30 minutes and centrifuged on a MicroCentaur bench top centrifuge for 5 minutes at 13 000 rpm. In the Bactopenase inhibition assay, the difference between the rate of Penicillin G degradation obtained when a culture supernatant sample was diluted with H_2O and when it was diluted with methanol represents the amount of inhibitory activity due to BLIP. BLIP activity is given as percent inhibition attributable to BLIP where the difference in rate of Penicillin G degradation is expressed as a percent of the rate obtained in the uninhibited reaction.

When methanol inactivation was the method used for removing proteinaceous β-lactamase inhibitory activity in culture supernatants, the uninhibited reaction also contained an equivalent amount of methanol as a control. The reaction components in this case consisted of 100 μl of the enzyme Bactopenase (2 x 10^4 units) diluted in 200 μl of phosphate buffer pH 7.0, 200 μl of supernatant diluted with methanol in a 1:1 (v/v) ratio or in the case of the uninhibited reaction, an equal volume of distilled H_2O and methanol. The components of the enzyme/inhibitor mixture were added in the above indicated order. Following incubation for 5 minutes at room temperature, the reaction was started by the addition of 500 μl of Penicillin G at 0.3 mg/ml.

II.9.2.2 Western immunodetection.

Protein concentrations of culture supernatants were quantitated by the Bio-Rad dye binding protein microassay. Sample preparation for SDS-PAGE was as described in Deutscher (1990). Five hundred microliters of culture supernatant was precipitated by the addition of an equal volume of methanol (spectro grade, Caledon Laboratories) or 125 μl of 50% TCA (Anachemia). The samples were incubated on ice for 30 minutes, centrifuged at 13 000 rpm in a bench top centrifuge and the pellet waswashed with acetone and redissolved in protein sample buffer (0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.025% Bromphenol Blue and 5% 2-mercaptoethanol) to a concentration of 1 μg/μl. The samples were incubated for 15 minutes at 95 °C and then subjected to 10 or 12% SDS-PAGE. The gel was blotted onto a PVDF membrane (Immobilon-P, Millipore Coorporation, Bedford, MA) as described by Pluskal *et al.* (1986). The membrane was wetted by rinsing with methanol then assembled in the Hoefer Transphor Tank transfer unit (Hoefer Pharmacia Biotech, San Francisco, California). Blotting was carried out at 1.5 amperes for 2 hours at 4 °C. Blotting buffer contained 25 mM Tris and 192 mM glycine at pH 8.3. Following transfer, the blot was rinsed with Tris buffered saline (TBS) containing 50 mM Tris base and 150 mM NaCl and adjusted to a pH of 7.5 with HCl and then blocked at 4°C overnight in blocking reagent [5% (w/v) skim milk in TBST (TBS containing 0.1% (v/v) Tween 20)]. Following blocking, western hybridization was carried out using the Boehringer Mannheim Chemiluminescence Western Blotting kit. The membrane was probed with primary anti-BLIP antibodies obtained from D. Alexander, Dept of Biological Sciences, University of Alberta, Edmonton, Alberta. Anti-BLIP antibody was used at a dilution of 1:4000 in 0.5 x blocking reagent. Incubation of the membrane with primary BLIP antibody was done for 1 hour at room temperature with gentle shaking. Following incubation with BLIP antibody, the membrane was washed twice for 10 minutes at room temperature with TSBT and then twice with 0.5 x TBST. The membrane was then incubated with gentle shaking at room temperature for half hour with peroxidase labelled anti-rabbit secondary antibody (anti-rabbit IgG-POD) diluted 1:5000 with 0.5 x blocking reagent. After probing with secondary antibody, the membrane was washed four times at room temperature with TBST. For chemiluminescence detection, the bound anti-rabbit IgG-POD catalyzes the oxidation of the diacylhydrazide substrate luminol resulting in the emission of light which was then detected by X-ray film. The PVDF membrane was exposed to the BM chemiluminesence substrate (30 minute preincubation of reagents A and B at a ratio of 100:1 according to manufacturer's instructions) at 20 µl of substrate solution per cm^2 of membrane area.

II.9.3 β-lactam assays

II.9.3.1 Bioassays.

TSBS culture supernatants were harvested from 24, 48, 72 and 96 hour cultures by centrifugation at 13 000 x g. The samples were spotted onto bioassay disks placed on TSB agar that had been inoculated with *E. coli* ESS. Bioassays were done on 9 x 9 inch bioassay plates. The TSB base agar was prepared as follows; 100 ml of molten TSB was cooled to 50 °C and 2 ml of a glycerol stock of *E. coli* ESS (OD_{600} of 2.41), a β-lactam supersensitive strain, was added and the plates left to solidify at room temperature in a laminar flow hood for 15 minutes. One half and one quarter inch Schleicher and Schuell bioassay disks were placed on the agar surface and 10-50 µl of culture supernatants were spotted on each disk. The plates were incubated overnight at 37 °C and zones of inhibition were measured.

II.9.3.2 Clavulanic acid and clavam assays by HPLC.

Imidazole derivatives of clavams and clavulanic acid were made by incubating 100 µl of culture supernatant with 25 µl of 0.18 mg/ml of imidazole reagent for 15 minutes at room temperature. Imidazole reagent contained 8.25 g of imidazole (Fisher) dissolved in 24 ml of deionized H_2O and 2 ml of 5 M HCl. The pH was adjusted to pH 6.8 with 5 M HCl and made to a final volume of 45 ml with distilled deionized H_2O. The reaction mixture was then analysed by HPLC on a µbondpack C-18 reverse phase column at 2 ml/min. The HPLC running buffer consisted of 0.1M HPLC grade NaH_2PO_4, (Fisher) and 6% spectro grade methanol (Caledon Laboratories). The pH was adjusted to 3.68 with HPLC grade glacial acetic acid (Fisher). Clavam-2-carboxylate and clavulanic acid were identified by comparison to retention times of authentic standards obtained from SmithKline Beecham, Worthing, England. Hydroxymethyl clavam was identified as the peak that ran between clavam-2-carboxylate and clavulanic acid (B. Barton, personal communication).

II.10 PENICILLIN G RESISTANCE STUDIES.

II.10.1 Surface culture studies

Assays were done in standard 100 x 15 mm petri plates. The base agar was 10 ml of trypticase soy and 1% starch medium containing 1.8% agar. When solidified, this was overlayed with 5 ml of soft TSBS agar inoculated with 1 x 10^7 viable spores and the plates were allowed to solidify for 20 minutes in a laminar flow hood.

II.10.1.1 Penicillin G resistance of ungerminated spores.

The overlaied plates prepared as described above were spotted with 1/2" Schleicher and Schuell bioassay disks and 50, 100 or 200 μg of Penicillin G was added to the disks. The plates were left in a laminar flow hood for 15 minutes for the absorption of the antibiotic onto the filter. The plates were then incubated at 28 °C and zones of inhibition were measured at 27 and 38 hours after inoculation.

II.10.1.2 Penicillin G resistance of germinated spores.

Plates containing TSBS base agar and soft TSBS agar overlay prepared as described in II.9 were incubated at 28 °C for six or sixteen hours. Bioassay disks were placed in the center of the plates and 50, 100 or 200 μg of Penicillin G was added. The plates were left in a laminar flow hood for 15 minutes after which they were incubated at 28 °C. Zones of inhibition were measured at 24, 37 and 45 hours after inoculation.

II.10.2 Liquid culture studies.

Duplicate 500 ml Erlenmeyer flasks containing 100 ml of TSBS medium were inoculated with a spore inoculum. At 12 hours after inoculation, Penicillin G was added to a final concentration of 0, 20 or 60 μg/ml. At 24 hour intervals, 150-600 μl samples were removed. Some of the sample was used for observation by phase contrast microscopy and the remaining part was homogenized with a disposable pellet pestle mixer (Kontes, Vineland, NJ) in order to determine the OD_{600} value.

II.11 PHASE CONTRAST MICROSCOPY.

From liquid cultures containing the various Penicillin G concentrations described in section II.9.2, 10 μl of a mycelial suspension was diluted with a drop of polyvinyl alcohol mounting medium, Salmon (1954), on a microscopic slide. The wet mount was analysed at 400 and 1000 x magnification by phase contrast microscopy.

II.12 GENETIC MAPPING OF THE *BLI* GENE.

II.12.1 PCR analysis.

The probes WS4 and WS5 were used for PCR amplification of the N-terminal half of the *bli* gene at a final concentration of 0.4 pmol/1 μl. They were designed by W.A. Schroeder (Department of Biological Sciences, University of Alberta) and have sequences 5' GAATTCATATGAGGACAGTGGGGATCGGCG 3' (WS4) and 5' GTTCCGGATCCTCAGGCCAGCAGCTTCTCCTGGCTC 3' (WS5). The annealing, extension and denaturation times and temperatures, optimised by W.A. Schroeder, were as follows: an initial denaturation step at 94 °C for 1' 30" and 29 cycles of amplification beginning with a denaturation step at 94 °C for 30 seconds, an annealing step at 60 °C for 1'15" and an extension step at 72 °C for 1'. The PCR was done with an MJ Research Minicycler. Taq polymerase was used in the amplification reaction. DNA templates consisted of preparations of the cosmids pBIP, NL1D1 and cosmid p53. DMSO concentrations were varied from 0-10% DMSO while Mg^{2+} concentrations varied from 0-8 mM $MgCl_2$. Results of reactions were separated on a 2% agarose gel. The positive control for the PCR reactions contained pBIP template DNA. In the pBIP positive control, 2 mM Mg^{2+}/7% DMSO, concentrations established in a previous experiment, were used to amply the half *bli* gene.

II.12.2 Mapping by Southern analysis.

Genomic DNA and the cosmids pBIP, p53 and NL1D1 were purified as described in section II.3. The probes used were the *bli* probe, the 4 kilobase left arm (L4) of p53 obtained by digestion of p53 with *Bgl* II/*Xba* I, the 3.3 kilobase right arm of p53 (R3.3) obtained by digestion of p53 with *Bam* HI/*Hind* III and an internal 2 kilobase *Bgl*II fragment (Bg-1.6) from the region to the right of the *cas1* region of p53 (Figure 8). These probes were labelled by nick translation as described in section II.8.2.1.

II.12.2.1 Cosmid hybridization.

The cosmids were digested with *Bgl* II/*Cla* I, *Hind* III, *Nco* I and *Sac* I. These four digests of p53, NL1D1 and pBIP were separated on a 0.4% agarose gel with *Pst* I digested lambda DNA standards. The gels were then subjected to acid depurination, blotted and the blots subjected to hybridization with the *bli* gene, L4 and R3.3 of cosmid p53, then washed and exposed to X-ray film as described in section II.8.2.2.

II.12.2.2 Genomic DNA hybridization.

Genomic DNA samples were simultaneously digested with *Bgl* II/*Cla* I, *Cla* I/*Sac* I, *Xho* I/*Cla* I, *Bgl* II/*Sac* I and *Bgl* II/*Xho* I. The DNA was run on a 0.5% agarose gel along side *Pst* I digested lambda DNA standards. The gels were blotted, the blots were hybridized with the *bli* gene, L4 and R3.3 fragment of cosmid p53, then washed and exposed to X-ray film as described in II.8.2.2.

II.12.2.3 Membrane stripping.

Hybridizing probes from previously probed nylon membranes were removed by incubation in a boiling solution of 0.1% (w/v) SDS. These were then left to cool at room temperature.

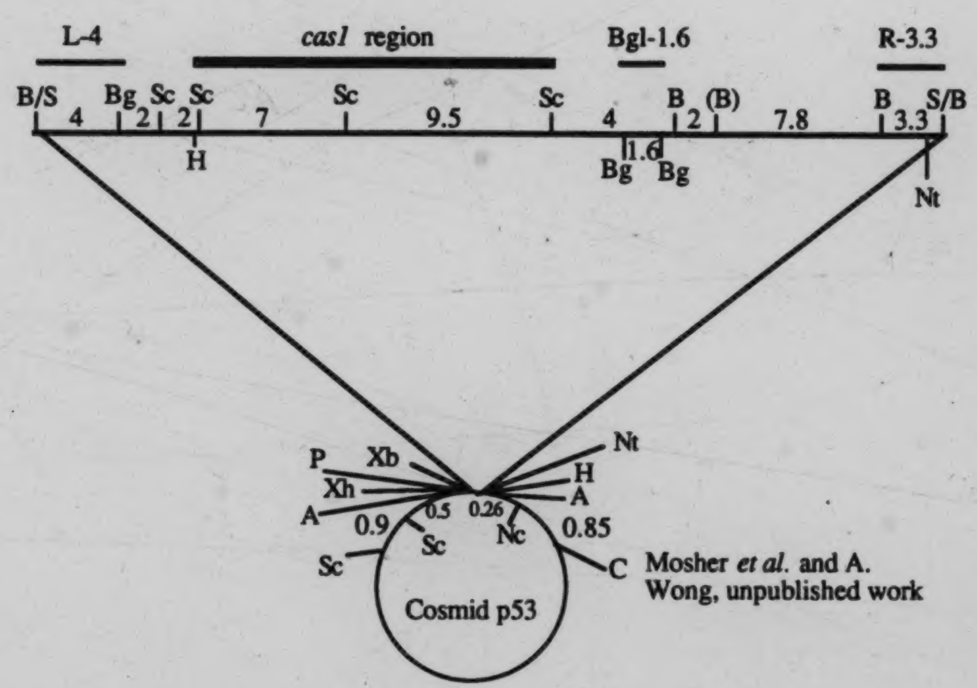
II.13 SEQUENCE ANALYSIS

II.13.1 Cosmid preparation.

pBIP was isolated from *E. coli* as described in section II.3. Uncut pBIP and *Nco* I digested pBIP were submitted to the Department of Biological Sciences DNA Sequencing Laboratory for sequencing. The primers designed for sequencing were also synthesized by the DNA Sequencing Laboratory, Department of Biological Sciences.

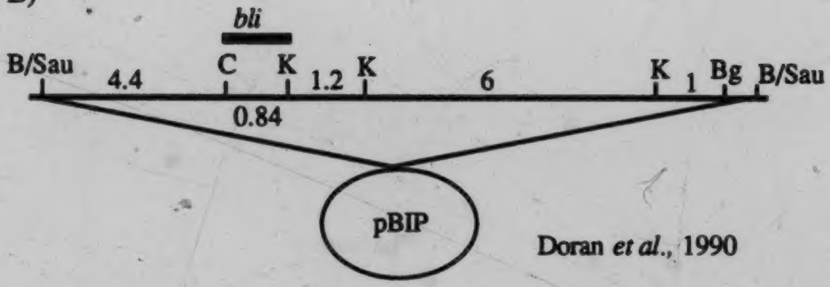
Figure 8. Location of probes from p53 and pBIP used in genetic mapping of *bli* gene. A) L-4, R-3.3 and Bg-1.6 are fragments from the insert in p53 (numeric values indicate length of fragments in kbs). L-4, the left end fragment of the insert in p53, was obtained from digestion of p53 with *Bgl* II/*Xba* I. R-3.3 is the right fragment of the p53 insert and was obtained by digestion of p53 with *Bam* HI/*Hind* III. Bg-1.6 is a ca. 1.6 kb internal fragment located in the area right of the *cas1* region in the p53 insert and was obtained by digestion of cosmid p53 with *Bgl* II. B) The *bli* probe is an 840 basepair *Cla* I/*Kpn* I fragment from the insert in pBIP. Abbreviations for the restrictions sites are as follows: A, *Ase* I; B, *Bam* HI; Bg, *Bgl* II; C, *Cla* I; H, *Hind* III; K, *Kpn* I; Nc, *Nco* I; Nt, *Not* I; P, *Pst* I; S, *Sal* I; Sc, *Sac* I; S/B, a *Sal* I and *Bam* HI hybrid site; and Xb, *Xba* I.

A)


L-4
cas1 region
Bgl-1.6
R-3.3
B/S
Bg
Sc
Sc
Sc
Sc
B
(B)
B
S/B
4
2
2
7
9.5
4
2
7.8
3.3
H
1.6
Bg
Bg
Nt
P
Xb
Xh
A
0.5
0.26
Nt
H
A
0.9
Sc
Nc
0.85
Sc
C
Cosmid p53

Mosher *et al.* and A. Wong, unpublished work

B)


bli
B/Sau
4.4
C
K
1.2
K
6
K
1
Bg
B/Sau
0.84
pBIP

Doran *et al.*, 1990

II.13.2 Sequencing primers.

Two sets of primers were made. Primers designed for sequencing downstream from the *bli* gene were designated as bli-n. These were designated "F" or "B" for forward, continuing downstream of the *bli* gene or backward on the complementary antisense strand respectively. Oligonucleotides designed to sequence downstream of *bliup1*, an open reading frame that is located upstream and divergent from the *bli* gene, were designated bliup1-n. These were also designated as "B" or "F" for backward or forward with respect to the *bliup1* gene. The locations of these primers with respect to the *bli* gene is shown in Figure 9.

Probes for sequencing downstream of the *bli* gene (shown in the 5' to 3'):

bli-1F: CCT CTC CTA CGG CGA TCA GC
bli-2F: TGC GGT TCG AGG GGC GCG TCA TC
bli-4F: GTG ATC CTG GTG ACC CAC GAC
bli-5F: GGA CGC GTA CGG CTC CCA CTC
bli-6F: GCA TCT TCC TCT CCG TCT G
bli-11F: GCG GGC CTG GGA ATC GTC TGC
bli-14F: GGT CGC GGG TGT GCT GCT CAC

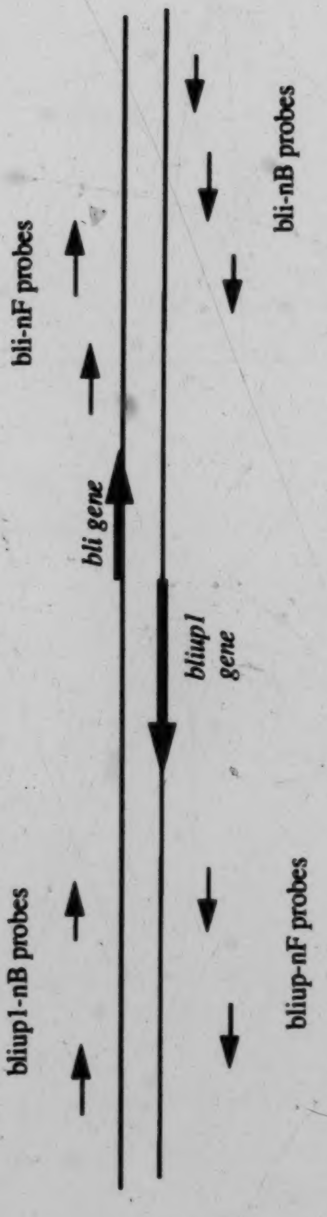
Probes for sequencing the antisense strand of the region downstream of the *bli* gene:

bli-3B: CAG TGA GCC GGT CGG CTC GTC
bli-6B: GTG AAC GAG TGG GAG CCG TAC
bli-7B: CGA ACC GTA CGT CCG TCA G
bli-8B: GTC GTG GGT CAC CAG GAT CAC
bli-9B: GCG ACC ACG AGG AGC GCG TTC
bli-10B: CGG GCA GCC GAT CAG ACA GAC
bli-13B: GCC GGG CAG CCG ATC AGA CAG
bli-15B: GAG CGA GTC CCC CAC GAG TAG
bli-16B: GGT CAG CTC GGG AAG CAA CTC

Probes for sequencing downstream of *bliup1*:

bliup1-1F: CCA TGT CCT CTT CCA AGC TGT
bliup1-2F: GCC CTG CTG CGC CTG TTC
bliup1-4F: GCA TCA CGG ATG ACC GCA TAG

Figure 9. Schematic diagram of oligonucleotides designed for sequencing regions in the vicinity of the *bli* gene. Dark divergent arrows represent the *bli* gene and *bliup1*, the open reading frame upstream of the *bli* gene. Directions of oligonucleotides synthesized for sequencing are indicated.


bli-nF probes
bli-nB probes
bli gene
bliup1 gene
bliup1-nB probes
bliup-nF probes

bliup1-6F: CCG GGC CGC TTC GGT CTC TC

Probes for sequencing the strand antisense to *bliup1*:

bliup1-3B: ATG CCG AGA TCA GCC GAT CCA GTT G
bliup1-5B: GTG TAT CCG TGG TGG TGC AC

II.13.3 Sequence Analysis.

Sequences of pBIP were obtained from Dept. of Biological Sciences Sequencing Laboratory and were compiled by DNA Star (DNASTAR, Inc., Madison, WI). Open reading frames were determined by codon preference of GCG (Genetics Computer Group, Inc. Madison, WI; or by PCG using a *Streptomyces* codon frequency table (Wright and Bibb, 1992). The sequences were also submitted to GCG/netBLAST search for amino acid sequence alignment.

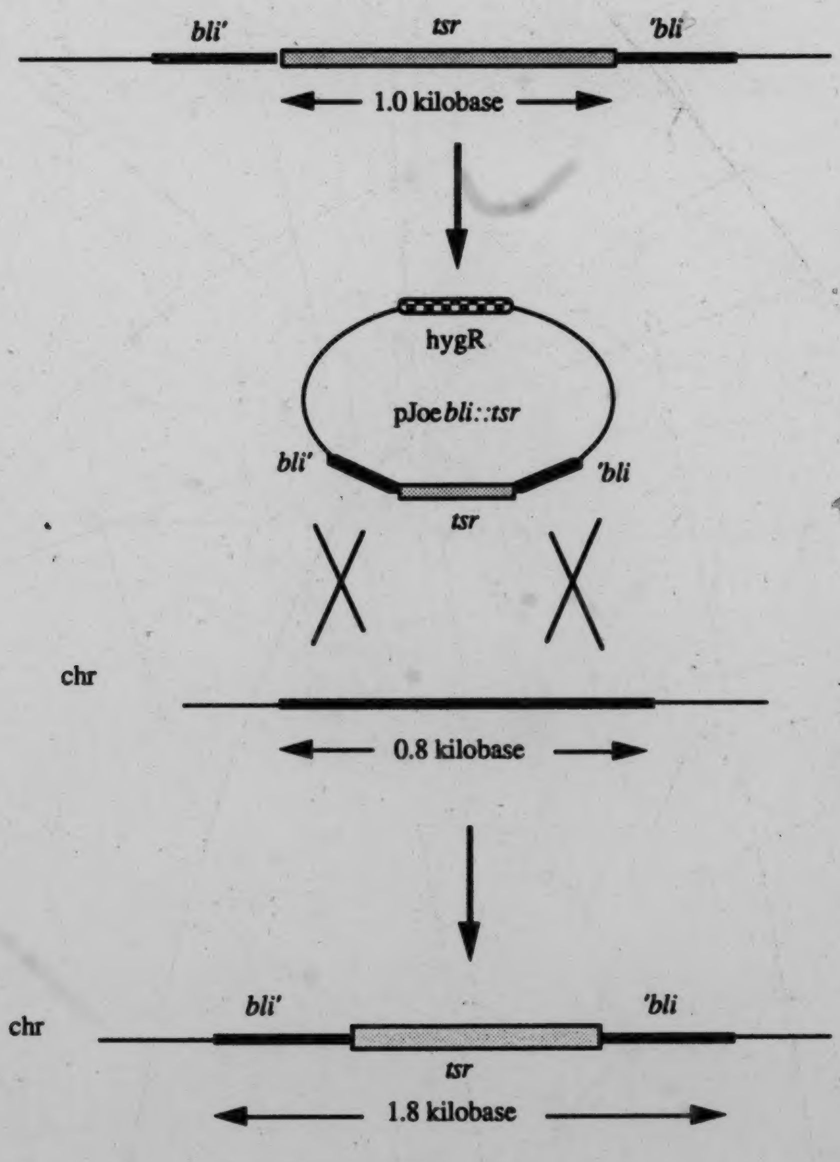
III. RESULTS: *BLI* GENE DISRUPTION

As part of the overall investigation of the role of BLIP in the growth and development of *S. clavuligerus*, it was desirable to create a mutant defective in BLIP production to assess what effects the absence of BLIP would have on the bacterium. Furthermore, since BLIP is one of two structurally different β-lactamase inhibitors produced by *S. clavuligerus*, a mutant defective in production of both inhibitors was also created in order to determine what role β-lactamase inhibitors play in the development of *S. clavuligerus*. This chapter summarizes experiments involved in creating the *bli* single mutant and the *bli*/*claR* double mutant and characterization of the resulting mutants. The single BLIP mutant and the clavulanic acid/BLIP double mutant were created by gene replacement at the *bli* locus in wild type *S. clavuligerus* and in a clavulanic acid production defective mutant, the *claR* mutant (Paradkar *et al.*, 1998), respectively. The mutants were assessed for their ability to produce β-lactam metabolites. The effect of the disruption on mycelial structure and sensitivity to Penicillin G was also studied.

III.1 DISRUPTION OF THE *BLI* GENE IN WILD TYPE *S. CLAVULIGERUS* AND IN THE CLAVULANIC ACID NONPRODUCER, THE *CLAR* MUTANT.

The 840 base pair *Cla I*/*Kpn* I fragment containing the *bli* gene, cloned in pUC119 (S. E. Jensen, unpublished), was disrupted by the insertion of a 1 kb thiostrepton resistant gene (*tsr*). The resulting *bli::tsr* disrupted gene (Figure 10) was placed into the *Streptomyces* vector, pJOE829. pJOE829 contains a hygromycin resistance gene (*hygR*) and therefore the presence of the plasmid can be determined by the hygromycin resistance phenotype. To create the *bli* single mutant, pJOE*bli::tsr*, the plasmid construct carrying the *bli::tsr* disrupted gene was first passed through *S. lividans* and then it was introduced into wild type *S. clavuligerus* where homologous double crossover at the *bli* locus occurred resulting in the replacement of the wild type *bli* gene with the *bli::tsr* disrupted gene. The *bli*/*claR* double mutant was created by introducing the same *bli::tsr* disruption construct from *S. lividans* into an existing *claR* single mutant. In each case, the passage of the plasmid construct through *S. lividans* presumably allowed for *Streptomyces* specific DNA modifications to take place so that

Figure 10. Strategy for the disruption of the *bli* gene by gene replacement. The *bli* gene located on an 840 bp fragment was disrupted by inserting a 1 kilobase thiostrepton resistance gene (*tsr*) into the BLIP coding sequence at an *Apa* I/*Bam* HI linker site generated at ca. 400 bp from the 5' end of the *bli* gene. The disrupted *bli* gene was cloned into *Streptomyces* vector pJOE829, which carries a hygromycin resistance gene (*hygR*). The resulting plasmid construct was called pJOE*bli*::*tsr*. When introduced into *S. clavuligerus*, replacement of the wild type *bli* gene on the chromosome (chr) with the disrupted *bli* gene of pJOE*bli*::*tsr* occurred through homologous recombination. Subsequent sporulation of the transformants in the absence of antibiotic selection resulted in loss of the plasmid. Clones which had undergone the double crossover event and plasmid curing via sporulation became thiostrepton resistant and hygromycin sensitive (tsr/hygS).



bli'
tsr
'bli
1.0 kilobase
hygR
pJoe bli::tsr
bli'
'bli
tsr
chr
0.8 kilobase
chr
bli'
'bli
tsr
1.8 kilobase

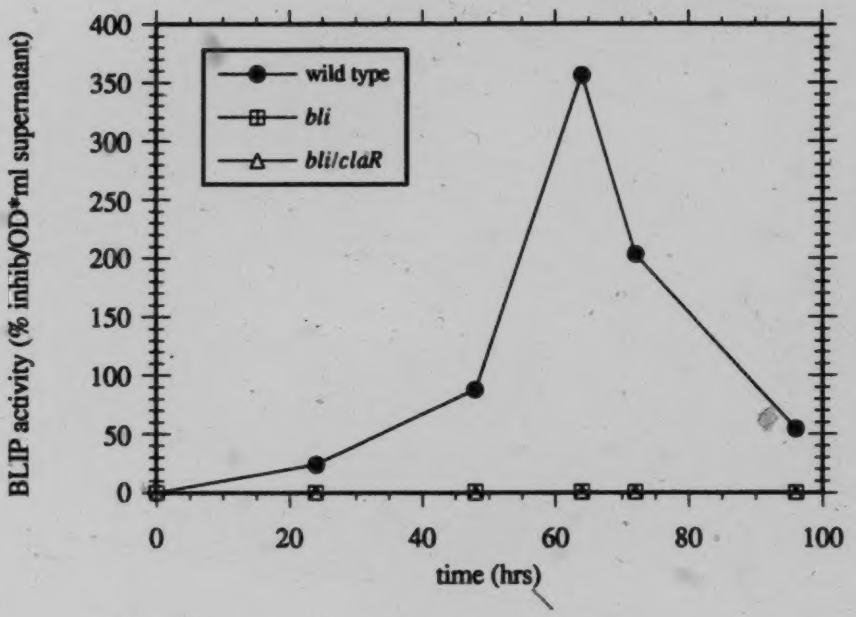
the resulting foreign DNA became less susceptible to the restriction systems of *S. clavuligerus*. DNA transformation procedures in which DNA from *E. coli* was used directly to transform *S. clavuligerus* were never successful. When pJOE*bli::tsr* was introduced into *S. clavuligerus* wild type and the *claR* mutant strain, the primary transformants were thiostrepton resistant (tsR) and hygromycin resistant (hygR). Twelve tsR/hyR primary transformants were obtained in wild type *S. clavuligerus* and 7 primary transformants were obtained in the *claR* mutant. Four of each strain were allowed to sporulate on nonselective media to induce plasmid loss and then the spores were harvested, serially diluted and plated for isolated colonies. In this process, most clones lost their plasmids during sporulation and became hygromycin sensitive and thiostrepton sensitive (tsS/hygS). The clones of interest were the small percentage of clones that had undergone homologous double crossover at the *bli* locus prior to plasmid curing. These were thiostrepton resistant and hygromycin sensitive (tsR/hyS); the hygS phenotype resulted from plasmid curing during sporulation. TsR/hygS clones were isolated by replica plating on thiostrepton containing media and hygromycin containing media. About 15 000 colonies from the four wild type primary transformant clones carrying the pJOE*bli::tsr* were screened for the tsR/hygS phenotype and seven putative *bli* single mutants were obtained. From the four *claR* primary transformants, about 60 000 colonies were screened and only one putative *bli/claR* double mutant was obtained. To determined if these putative mutants were BLIP production defective mutants, culture supernatants of the seven single *bli* mutants and the *bli/claR* double mutant grown in TSBS were assayed for BLIP activity by β-lactamase inhibition assay as described in Materials and Methods. Figure 11 shows BLIP activity produced by wild type, one representative single *bli* mutant and the *bli/claR* double mutant. Although high levels of BLIP activity were seen in the wild type culture supernatants, at no point in the time course was BLIP seen in either the single or double *bli* mutants confirming that the tsR/hygS mutants were true BLIP mutants and were defective in BLIP production.

III.2 CONFIRMATION OF GENE REPLACEMENT BY SOUTHERN HYBRIDIZATION.

Southern hybridization was carried out to confirm that the gene disruption event had taken place at the *bli* locus in the genome of the BLIP nonproducers isolated by replica

Figure 11. Time course comparison of BLIP activity in TSBS cultures of wild type and mutant strains of *S. clavuligerus*. TSBS cultures of wild type *S. clavuligerus*, the *bli*, and *bli/claR* mutants were harvested at 24, 48, 64, 72 and 96 hours. BLIP activity assay results are shown as % inhibition/OD_{600}*ml of supernatant.

Time course analysis of BLIP production



plating. Genomic DNA of wild type, the single and double mutants was isolated as described in Hopwood et al. (1985). DNA preparations were digested with *Kpn* I and separated by agarose gel electrophoresis. The gel was blotted onto nylon membrane by the method of Southern (1975). The membranes were probed with an 840 basepair fragment containing the *bli* gene and a 1.09 kb fragment containing the *tsr* gene, both labeled with α^{32}P-dCTP by nick translation (Figure 12). When probed with the labeled *bli* gene, a 5.5 kb *Kpn* I fragment was seen in the wild type strain (Figure 12 A). This was replaced with a 6.5 kb *Kpn* I fragment in the DNA of the single *bli* mutant and *bli/claR* double mutant suggesting that the *Kpn* I fragment carrying the *bli* gene had taken up a 1 kb insert, presumably the *tsr* gene. When probed with a labeled *tsr* gene, (Figure 12 B), no signal was seen in wild type genomic DNA digests as expected since wild type *S. clavuligerus* does not have a *tsr* gene. In the DNA digest of the *bli* mutant and the *bli/claR* double mutant however, a band of 6.5 kb was also seen. This confirmed that the gene replacement event had taken place in the *bli* and *bli/claR* mutants

III.3 CHARACTERIZATION OF THE *BLI* AND *BLI/CLAR* MUTANTS.

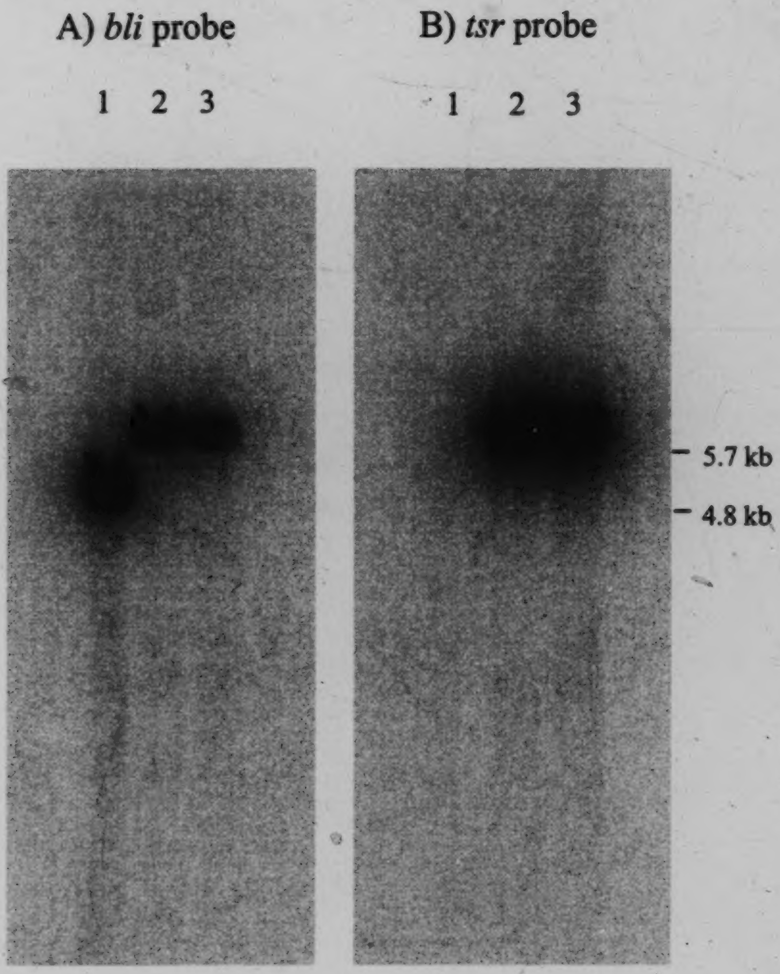
The effects of the *bli* disruption on the following aspects of *S. clavuligerus* biology were examined:

- Production of clavulanic acid, clavams and cephamycin.
- Susceptibility to Penicillin G in liquid medium and surface medium-grown cultures.
- Morphology of liquid medium-grown mycelia.

III.3.1 Comparisons of clavulanic acid and clavam levels in the *bli* single mutant and the *bli/claR* double mutant with wild type and the *claR* single mutant

Clavulanic acid and the two clavams detectable by HPLC, clavam-2-carboxylate and hydroxymethyl clavam, are produced in abundance in soy fermentation medium by 72 and 96 hours in cultures of wild type *S. clavuligerus*. To determine if the *bli* disruption had any effect on clavulanic acid, clavam-2-carboxylate and hydroxymethyl clavam production, culture supernatants from triplicate soy cultures of the *bli* single mutant, the

Figure 12. Southern analysis of *S. clavuligerus bli* and *bli/claR* mutants for the gene disruption event. Total DNA was isolated from wild type *S. clavuligerus*, the *bli* mutant and the *bli/claR* double mutant. The DNA was digested by *Kpn* I, separated on 0.8 % agarose gels and blotted onto nylon membranes. Duplicate blots A and B were probed with the *bli* probe, an 840 basepair fragment containing the *bli* gene, (blot A), and the *tsr* probe, 1.09 kilobase fragment containing the *tsr* gene, (blot B). Both probes were labelled with α-^{32}P by nick translation. Lanes 1 in blots A and B contain *Kpn* I digested DNA from wild type *S. clavuligerus*, lanes 2 and 3 in blots A and B contain DNA from the *bli* single mutant and the *bli/claR* double mutant respectively.


A) *bli* probe
1
2
3
B) *tsr* probe
1
2
3
5.7 kb
4.8 kb

bli/*claR* double mutant, the *claR* single mutant and wild type *S. clavuligerus* were analysed by HPLC.

Comparisons of clavulanic acid levels produced by the wild type and single mutant showed no apparent differences at 48, 72 or 96 hours (Table 2). The disruption of the *bli* gene seems to have no effect on clavulanic acid production. Neither *claR* nor the *bli*/*claR* mutant produced any clavulanic acid as expected since the *claR* disruption occurred in a transcriptional activator gene important for the expression of enzymes in the later steps of the clavulanic acid biosynthetic pathway (Paradkar *et al.*, 1998).

Clavam-2-carboxylate peaks were identified by running authentic standards while hydroxymethylclavam was identified as the peak that ran between clavam-2-carboxylate and clavulanic acid. The levels of clavam-2-carboxylate in the culture supernatants of wild type *S. clavuligerus*, *bli*, *claR* and *bli*/*claR* are shown in Table 3. At 48 hours, no apparent difference in clavam-2-carboxylate was seen in the culture supernatants of the *bli* mutant when compared to that of wild type *S. clavuligerus*. At 72 hours, the *bli* mutant produced levels of clavam-2-carboxylate that were two to five times higher than that seen in the wild type samples. Although the *bli*/*claR* double mutant showed a lag in production of this clavam at 48 hours, by 72 hours, more clavam-2-carboxylate was detected in the culture supernatants of the *bli*/*claR* double mutant than in the *claR* single mutant or in wild type culture supernatants. This difference was transient and was not detected in the 96 hour samples. There was one sample out of the six replicates of each strain in the two time courses, that did not seem to support this observation.

Levels of hydroxymethyl clavam are shown in Table 4. As seen with clavam-2-carboxylate, no difference between wild type and the *bli* mutant was detected in the 48 hour samples. At 72 hours, culture supernatants of the *bli* mutant showed levels of hydroxymethyl clavam that were two to almost ten fold higher than in the wild type samples. The data for the 96 hour samples were more variable yet four of the highest levels of hydroxymethyl clavam detected were that from the *bli* culture supernatants. Although the *bli*/*claR* double mutant also showed an initial lag in hydroxymethyl clavam production at 48 hours, at 72 and 96 hours, comparisons of the *claR* single mutant versus the *bli*/*claR* double mutant showed that both strains were producing wild type levels of this clavam.

Although there were a few exceptions, the general pattern seems to be that in cases where there were differences in clavam levels among the mutants, either clavam-2-carboxylate or hydroxymethyl clavam, the higher clavam level was seen in the *bli* single

Table II. Production of clavulanic acid by cultures of wild type *S. clavuligerus*, the *bli*, *claR* and *bli/claR* mutants grown in soy medium for 48, 72 and 96 hours.

	48 hour samples		72 hour samples			96 hour samples		
	DNA[b] (mg/ml)	clavulanic acid[c] (µg/mg DNA)	DNA[b] (mg/ml)	clavulanic acid[c] (µg/mg DNA)		DNA[b] (mg/ml)	clavulanic acid[c] (µg/mg DNA)	
Strains[a]				tc-1	tc-2		tc-1	tc-2
wild type								
1	0.085	143	0.091	320	361	0.114	132	334
2	0.089	130	0.083	420	342	0.094	178	375
3	0.083	174	0.093	275	371	0.090	278	221
bli								
1	0.043	346	0.084	434	297	0.092	197	297
2	0.057	349	0.085	334	261	0.102	213	283
3	0.083	144	*0.082	263	560	0.064	226	235
claR								
1	0.070	1.7	0.109	0.228	0	0.131	0.352	2.11
2	0.101	0	0.113	0	0	0.130	0.247	5.00
3	0.084	0	0.111	0.228	0	0.128	0	0.922
bli/claR								
1	0.092	0	*0.088	1.56	0.931	0.124	0.352	1.6
2	0.096	0	*0.086	0	4.76	0.145	0.666	5.16
3	0.080	0	0.102	0.762	2.85	0.113	0.457	4.15

[a] Triplicate cultures of each strain were analysed for clavulanic acid content. Two time course experiments (tc-1 and tc-2) were done for the 72 and 96 hour samples. The asterisks mark the averages of duplicate values which differed by more than 0.05 mg/ml.

[b] DNA contents (mg of DNA /ml of culture) were determined by the Burton assay as described in Materials and Methods.

[c] Clavulanic acid amounts (µg/mg DNA) were quantitated by HPLC and then normalized for growth relative to DNA contents.

Table III. Production of clavam-2-carboxylate in cultures of wild type *S. clavuligerus*, *bli*, *claR*, and *bli/claR* mutants grown in soy medium for 48, 72 and 96 hours.

	48 hour samples		72 hour samples			96 hour samples		
	DNA[b]	clavam-2-carboxylate[c] (µg/mg DNA)	DNA[b]	clavam-2-carboxylate[c] (µg/mg DNA)		DNA[b]	clavam-2-carboxylate[c] (µg/mg DNA)	
Strains[a]	mg/ml		mg/ml	tc-1	tc-2	mg/ml	tc-1	tc-2
wild type								
1	0.085	87.3	0.091	231	222	0.114	612	791
2	0.089	105	0.083	789	210	0.094	853	355
3	0.083	101	0.093	250	198	0.090	459	234
bli								
1	0.043	99.8	0.084	751	834	0.092	743	823
2	0.057	118	0.085	1656	753	0.102	1093	148
3	0.083	33.3	*0.082	1502	1216	0.064	700	1214
claR								
1	0.070	116	0.109	520	510	0.131	754	421
2	0.101	102	0.113	520	383	0.130	241	844
3	0.084	149	0.111	289	365	0.128	612	804
bli/claR								
1	0.092	13.5	*0.088	1925	704	0.124	667	1427
2	0.096	14.6	*0.086	1791	532	0.145	743	498
3	0.080	54	0.102	1271	807	0.113	951	726

[a] Triplicate cultures of each strain were grown for HPLC analysis of clavam-2-carboxylate contents. Two time course experiments (tc-1 and tc-2) were done for the 72 and 96 hour samples. Asterisks mark averages of duplicate values that differed by > 0.05 mg/ml.

[b] DNA contents were determined by the Burton assay using salmon DNA standards and are given in mg of DNA/ml of culture.

[c] Clavam-2-carboxylate amounts (µg/mg DNA) were quantitated by HPLC and normalized relative to DNA contents of the respective cultures.

Table IV. Production of hydroxymethyl clavam in cultures of wild type *S. clavuligerus*, the *bli*, *cla* and *bli/cla* mutants grown in soy medium to 48, 72 and 96 hours.

	48 hour samples		**72 hour samples**			**96 hour samples**		
	DNA[b] (mg/ml)	hydroxymethyl clavam[c] (µg/mg DNA)	DNA[b] (mg/ml)	hydroxymethyl clavam[c] (µg/mg DNA)		DNA[b] (mg/ml)	hydroxymethyl clavam[c] (µg/mg DNA)	
Strains[a]				tc-1	tc-2		tc-1	tc-2
wild type								
1	0.085	45.4	0.091	92.1	80.4	0.114	436	508
2	0.089	51.8	0.083	236	69.0	0.094	608	197
3	0.083	46.3	0.093	84.1	72.8	0.090	218	193
bli								
1	0.043	96.4	0.084	396	667	0.092	1983	725
2	0.057	122	0.085	1388	782	0.102	1312	126
3	0.083	41.8	*0.082	1012	652	0.064	643	1273
cla								
1	0.070	92.8	0.109	212	227	0.131	477	206
2	0.101	83.4	0.113	291	134	0.130	148	636
3	0.084	120	0.111	145	131	0.128	613	427
bli/cla								
1	0.092	8.98	*0.088	376	278	0.124	375	1035
2	0.096	8.07	*0.086	434	164	0.145	364	347
3	0.080	49.9	0.102	190	238	0.113	475	562

[a] Triplicate cultures of each strain were grown for determination of hydroxymethyl clavams levels. Two time course experiments (tc-1 and tc-2) were done for the 72 and 96 hour samples. Asterisks mark the averages of duplicate values that differed by > 0.05 mg/ml.

[b] DNA contents of each culture were determined by Burton assays and are given as mg of DNA/ml of culture.

[c] Levels of hydroxymethyl clavams (µg clavulanic acid equivalents/mg DNA) were determined by HPLC. Values were normalized relative to DNA content of cultures.

or double mutant. The large variation seen in amounts of clavams (and clavulanic acid) produced by different cultures seem to be an innate feature of these experiments and suggests that β–lactam production is highly sensitive to growth conditions and will vary greatly in response to differences in the different flasks/volume of media within each flask among replicate experiments. Furthermore this difference was seen even when DNA measurements indicated similar extents of growth.

III.3.2 Effect of the *bli* disruption on cephamycin production in the *bli* single mutant and the *bli*/*claR* double mutant.

Cephamycin production by wild type *S. clavuligerus*, the *bli* single mutant and the *bli*/*claR* double mutant was determined by bioassay against the indicator organism *E. coli* ESS. Wild type *S. clavuligerus* and the various mutants were grown in TSBS for 48, 72 and 96 hours. No difference in the level of antibiotic production was found among the mutants and wild type *S. clavuligerus*. (Data not shown.)

III.3.3 Comparison of Penicillin G sensitivity in liquid and surface cultures.

Although no difference was seen in antibiotic production among the mutants and wild type *S. clavuligerus*, it seemed possible that BLIP might contribute the high β-lactam resistance of *S. clavuligerus*. Penicillin G was the antibiotic of choice for these studies as *S. clavuligerus* is most sensitive to this particular antibiotic. Since liquid medium-grown and surface-grown cultures may show different sensitivity to Penicillin G, sensitivity studies were done with liquid and surface grown cultures.

III.3.3.1 Penicillin G sensitivity of surface cultures.

The sensitivity of the *bli*, *claR* and *bli*/*claR* mutants and wild type *S. clavuligerus* to Penicillin G was determined by bioassay. Each bioassay plate was overlayed with spores of the wild type or mutant *S. clavuligerus* strains and tested for sensitivity to Penicillin G. No significant difference in Penicillin G susceptibility among the different strains was seen. Results are not shown.

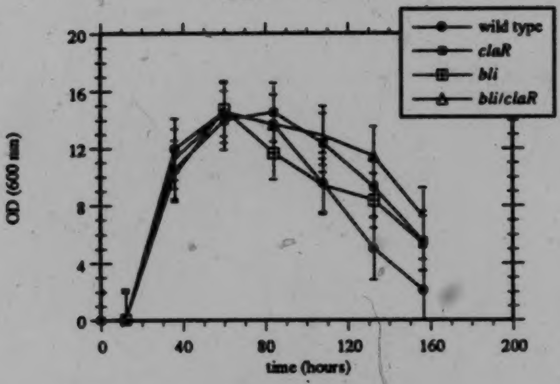
III.3.3.2 Penicillin G sensitivity in liquid cultures.

Wild type *S. clavuligerus* and the three mutants were grown in 100 ml amounts of TSBS broth from a spore inoculum. At twelve hours post inoculation, Penicillin G was added to these flasks to a final concentration of 0, 20 and 60 µg/ml. Figure 13 shows growth curves of wild type *S. clavuligerus* and the various mutants in the presence of 0, 20 and 60 µg/ml of Penicillin G as indicated. There were no apparent and reproducible difference was observed among the different mutants and wild type *S. clavuligerus*.

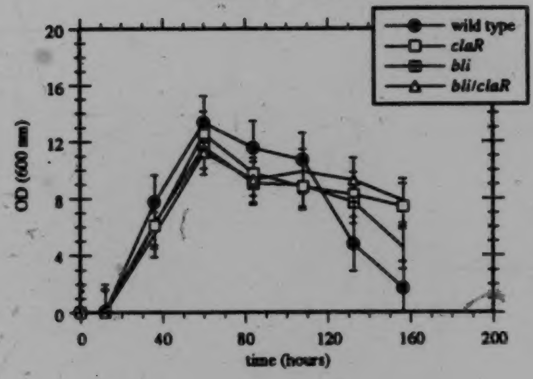
III.3.4 Effect of the *bli* disruption on morphology.

Samples of wild type *S. clavuligerus* and the mutant strains used in the above experiment were analysed by phase contrast microscopy. A mycelial suspension from TSBS cultures, prepared for phase contrast microscopy as described in Materials and Methods was photographed at 400 and 1000 x magnifications. No morphological differences were seen among the different mutants and the wild type organism (data not shown). Sporulation was also unaffected by the *bli* disruption.

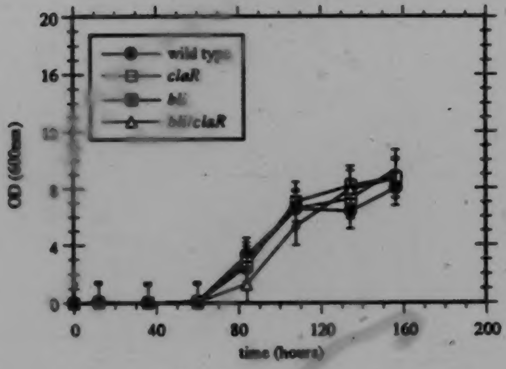
Figure 13. Sensitivity of *S. clavuligerus* wild type and mutant strains to Penicillin G in liquid cultures. Wild type *S. clavuligerus* , the *bli* mutant, the *claR* mutant, and the double mutant *bli/claR* grown in the absence of Penicillin G, at 20 µg/ml of Penicillin G, and at 60 µg/ml of Penicillin G. The data plotted are averages of duplicate cultures.



0 µg/ml Penicillin G



20 µg/ml Penicillin G



60 µg/ml Penicillin G

IV. RESULT: GENETIC MAPPING OF THE *BLI* GENE

Genetic mapping of the *bli* gene by PCR and Southern hybridization experiments involving cosmid DNA and genomic DNA was done to determine its location with respect to known β-lactam biosynthetic genes. Due to the phenomenon of gene duplication in *S. clavuligerus* and the fact that a gene coding for BLP exists within the cephamycin/clavulanic acid gene cluster, the search for the location of the *bli* gene was directed toward the other β-lactam cluster, the clavam biosynthetic gene cluster. Two cosmids containing clavam genes were used in these mapping studies. NL1D1 is an unstable cosmid isolated from a *S. clavuligerus* library by R. H. Mosher, N. Logsetti and S. E. Jensen (unpublished result) in a collaborative project in the lab. The cosmid contained at least six clavam genes, one of which is a homolog of a gene located in the clavulanic acid biosynthetic pathway. NL1D1 was found to be unstable as different preparations of the cosmid yielded multiple deleted forms. A stable derivative of NL1D1, called cosmid p53, was then isolated by A. Wong by introduction of NL1D1 into *E. coli* DH5α and screening for the largest stable clone. A restriction map of cosmid p53 as determined by A. Wong and A. Paradkar. (unpublished result) is given in Figure 8 A (Section II.12.2). The 7 and 9.5 kb *Sac* I fragments labeled as the *cas1* region were believed to be contiguous and representive of the genome (R. H. Mosher, C. Anders and S. E. Jensen (unpublished result). It was not certain whether the other regions were contiguous or how the insert in p53 compared to NL1D1. The NL1D1/p53 plasmid vector pFD666, is an *E. coli/Streptomyces* shuttle vector of approximately 5.25 kbs. The insert in NL1D1 is cloned into a *Bam* HI site in the multiple cloning site. Relevant restriction sites in pFD666 are shown in Figure 8, Section II.12.2.

IV.1 PCR EXPERIMENTS TO DETERMINE THE LINKAGE OF THE *BLI* GENE TO GENES INVOLVED IN CLAVAM BIOSYNTHESIS.

IV.1.1 PCR Experiments to Locate the *Bli* Gene on the Clavam Cosmid NL1D1.

To determine whether the *bli* gene was located near clavam genes, attempts were made to amplify the *bli* gene from the clavam cosmid NL1D1 using PCR. The set of primers used, (WS4 and 5), was designed by W. Schroeder (University of Alberta, Edmonton, Alberta) to generate the N-terminal half of the *bli* gene, a fragment of approximately 300 basepairs. pBIP (Doran *et al.*, 1990), a cosmid carrying an insert of 13.5 kbs from which the *bli* gene was originally cloned (Figure 8 B, Section II.12.2), was used as a positive control for the PCR reaction. Cosmid preparations and PCR amplification conditions were as described in Materials and Methods. Lane 16 in Figure 14 A shows the N-terminal half of the *bli* gene, amplified by WS4 and WS5 from pBIP, the positive control. Lanes 9 and 13 which corresponded to reactions containing Mg^{2+}/DMSO amounts of 2 mM/7% and 2 mM/10% show PCR products of a similar size when NL1D1 was the template DNA. This product was purified from the PCR reaction by PEG precipitation (see Materials and Methods) and sequenced using the PCR primers WS4 and WS5. The sequence was found to be that of the N-terminal half of the *bli* gene. Although it was uncertain whether NL1D1 contained the full *bli* gene, it was clear that the N-terminal half of the gene was present on this clavam cosmid. It was unlikely that the half *bli* gene obtained from PCR amplification resulted from genomic DNA contamination since the cosmids NL1D1, p53 and pBIP were prepared from *E. coli* hosts which do not have a *bli* gene.

IV.1.2 PCR to Determine if p53 Contained the *Bli* Gene.

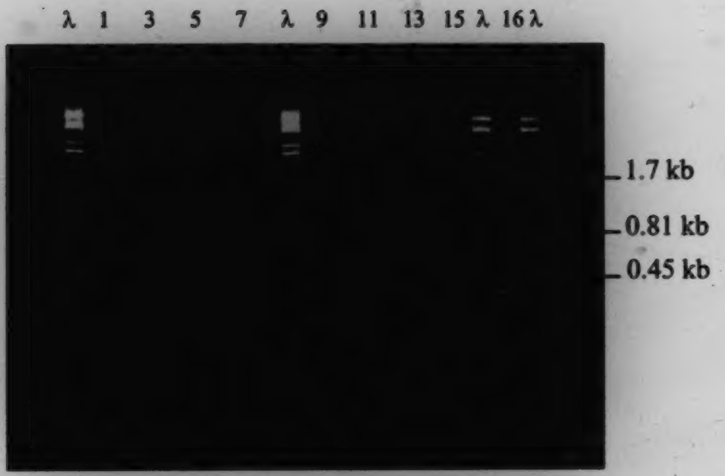
Since p53 was derived from NL1D1, PCR experiments using p53 as the DNA template were done to determine if p53 also contained the *bli* gene. The primers were WS4 and WS5, the same oligonucleotides used to amplify the *bli* gene from NL1D1 in the PCR experiment described above. Cosmid DNA preparations and amplification conditions were as above. As shown in lane 1, Figure 14 B, the half *bli* gene can be amplified from pBIP, the positive control. No product was obtained in reactions where p53 was used as the DNA template under any concentrations of Mg^{2+} and DMSO (lanes 2-16). The absence of *bli* on p53 was presumed to be a result of deletion(s) and or rearrangements that had taken place in the original NL1D1 cosmid clone prior to transformation into *E. coli* DH5α.

IV.2 CONFIRMATION OF THE PRESENCE OF THE *BLI* GENE ON NL1D1 BY SOUTHERN HYBRIDIZATION.

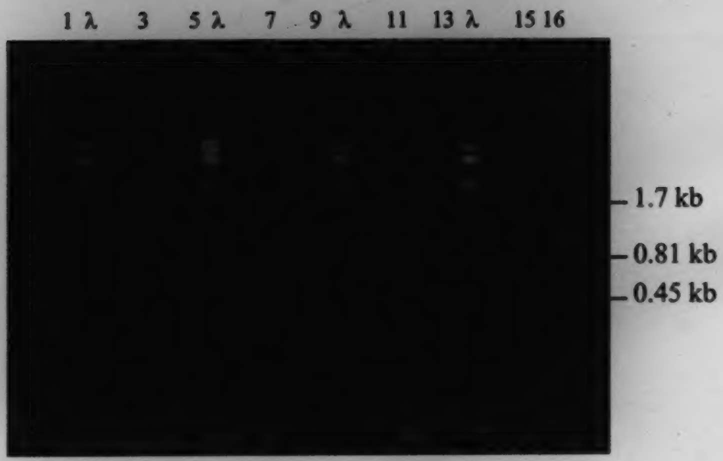
IV.2.1 Hybridization of Various Digests of NL1D1 DNA with the *bli* probe.

Figure 14. PCR amplification of the N-terminal half of the *bli* gene from the clavam cosmids NL1D1 (A) and cosmid p53 (B). The primers WS4 and WS5 were used to amplify the half *bli* gene by Taq DNA polymerase. The positive controls, gel A/lane 16 and gel B/lane 1 represented results obtained when the *bli* gene containing cosmid pBIP was used as the template for PCR in the presence of 2 mM Mg^{2+}/5 % DMSO. The negative controls (gel A/lane 1 and gel B/lane 2) are results from PCRs containing 0% DMSO/0 mM Mg^{2+}. The remaining PCRs were done at 2 to 10% DMSO and 2 to 8 mM Mg^{2+}. [Gel A (NL1D1 template): lanes 2-4, 2% DMSO/2, 4, or 6 mM Mg^{2+}; lanes 5-8, 4.5% DMSO/2, 4, 6 or 8 mM Mg^{2+}; lanes 9-12, 7% DMSO/2, 4, 6 or 8 mM Mg^{2+}; and lanes 13-15, 10% DMSO/2, 4 or 6 mM Mg^{2+} respectively. Gel B (p53 template): lanes 3-5, 2% DMSO/4, 6 or 8 mM Mg^{2+}; lanes 6-9, 4.5% DMSO/2, 4, 6, or 8 mM Mg^{2+}; lanes 10-13, 7% DMSO/2, 4, 6 or 8 mM Mg^{2+}; and lanes 14-16, 10% DMSO/2, 4, 6 mM Mg^{2+} respectively.] In gel A, lanes 9 and 13, a PCR product the same size as half *bli* in the positive control was seen. Lanes labeled as λ contained λ DNA digested with *Pst* I.

A.


λ 1 3 5 7 λ 9 11 13 15 λ 16 λ
1.7 kb
0.81 kb
0.45 kb

B.


1 λ 3 5 λ 7 9 λ 11 13 λ 15 16
1.7 kb
0.81 kb
0.45 kb

In the initial confirmation that NL1D1 contained clavam genes, A. Paradkar prepared a Southern blot of various digests of NL1D1 that was probed with a gene from the clavam cluster. This blot was stripped and reprobed with the *bli* gene as described in Materials and Methods (II.12.2.3). Hybridizing bands of two different sizes were seen (Figure 15). A similar high molecular weight band was seen in *Pst*I (lane 4), *Xba* I (lane 5), and *Pst* I/*Xba* I (lane 7) digests of NL1D1. These were believed to be signals arising from linearized vector (see Figure 7 for map of p53/NL1D1). A second band of approximately 5 kbs was seen in the lanes in which NL1D1 was digested with *Nco* I, *Pst* I/*Nco* I and *Xba* I/*Nco* I. Since this 5 kb band was seen only in the single *Nco* I digest and in double digests in which one enzyme was *Nco* I, it was concluded that *bli* is located on a 5 kb *Nco* I fragment in NL1D1.

IV.2.2 Comparison of NL1D1 and Cosmid p53 Inserts by *Ase* I Digestion.

The inability to amplify the *bli* gene from p53 by PCR indicated that cosmid p53 did not contain the original insert cloned in NL1D1. The *bli* gene was believed to be located on sequences that, although initially present on NL1D1, were either deleted or rearranged during the formation of the stable cosmid p53. To determine if the insert in cosmid p53 had arisen from deletions in the NL1D1 insert, cosmid preparations of p53 and NL1D1 were digested with *Ase* I. *Ase* I sites flank the cloned insert so digestion with *Ase*I would release the insert from the cosmid vector. Figure 16 A shows *Ase* I digests of NL1D1 and p53 DNA separated on an agarose gel. Analysis of the *Ase* I digested NL1D1 preparation showed that in addition to the vector fragment, three insert fragments of 16, 22 and greater than 27 kbs were seen. [Since the length of insert between two cos sites are expected to be 38-55 kb, 78%-105% wild type, the insert would be expected to be 33-50 kbs as the plasmid vector is 5.2 kbs. *Sal* I DNA fragments of 30-40 kb were used for library construction, R.H. Mosher N. Logsetti and S. E. Jensen (unpublished result).] At lower DNA concentrations, the largest fragment appeared to be a dimer in the original gel although this is difficult to see in the reproduced figure. The *Ase* I digested p53 preparation at 10 fold dilution (lane 6), appeared to consist of only one fragment in addition to the vectort. This fragment migrated to a similar position with that of the dimeric insert in NL1D1.

It was concluded that the NL1D1 cosmid preparation appeared to be composed of at least four clones containing inserts that were 16 and 22 kbs and possibly two large but

Figure 15. Southern hybridization of various digests of NL1D1 with the 840 basepair *bli* probe. Lane 1 contained lambda DNA standards in the original agarose gel, lane 2 is a blank while lanes 3 through 8 contained digests of NL1D1 DNA. Lanes 3, 4 and 5 contained NL1D1 DNA digested with *Nco* I, *Pst* I and *Xba* I, respectively. Lanes 6, 7 and 8 contained NL1D1 DNA doubly digested with *Pst* I/*Nco* I, *Xba* I/*Pst* I and *Xba* I/*Nco* I, respectively.

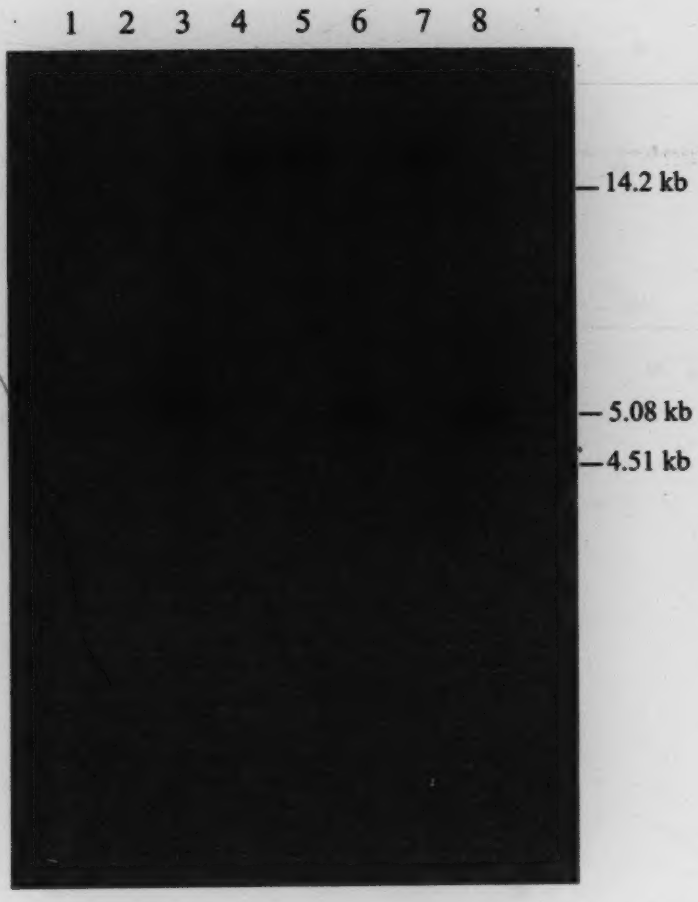
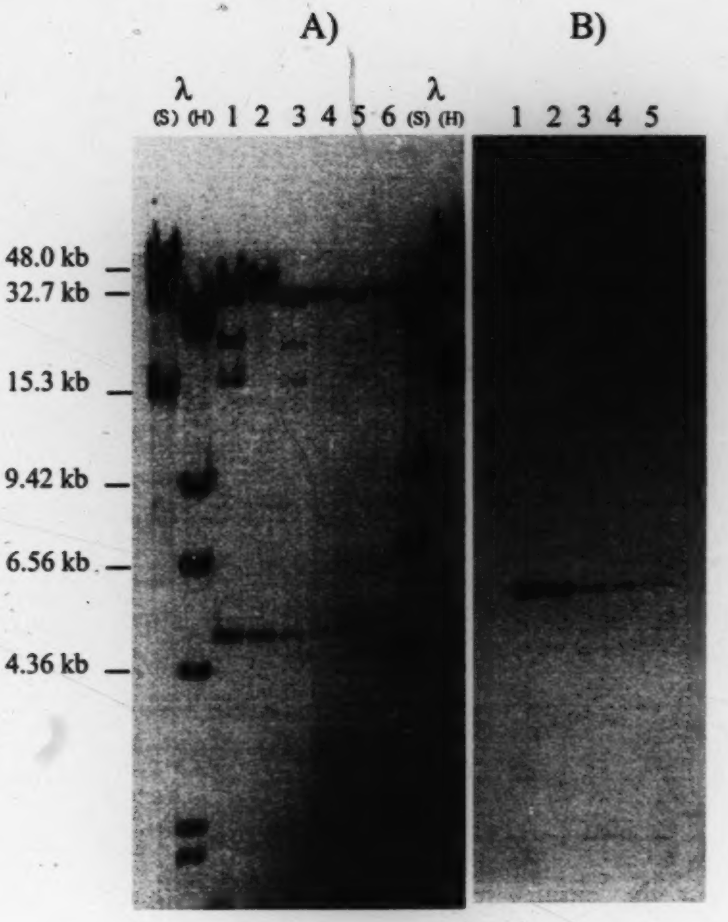

1 2 3 4 5 6 7 8
14.2 kb
5.08 kb
4.51 kb

Figure 16. Analysis of NL1D1 and cosmid p53 inserts by agarose gel electrophoresis (A) and Southern hybridizations (B). NL1D1 and cosmid p53 DNA preparations were digested with *Ase* I to separate the inserts from the vector pFD666. The digests were separated on a 0.5 % agarose gel, the gel was stained with ethidium bromide and photographed (A). Molecular weight size markers are lambda DNA (λ) digested with *Sal* I (S) and *Hind* III (H). Lanes 1, 3, and 5 contained 10, 2 and 1 µl of *Ase* I digested NL1D1 DNA respectively. Lanes 2, 4, and 6 contained 10, 2 and 1 µl of *Ase* I digested cosmid p53 DNA respectively. In B), *Ase* I digested NL1D1 and p53 cosmid DNA were hybridized with the 840 base pair *bli* probe. Lanes 1 and 4 contained *Ase* I digested NL1D1 DNA, undiluted and diluted ten fold, respectively. Lanes 2 and 5 contained *Ase* I digested p53 DNA undiluted and ten fold diluted respectively. Bands corresponding to the linearized plasmid vector are indicated by the arrow.


A)
B)
λ
(S) (H) 1 2 3 4 5 6
λ
(S) (H)
1 2 3 4 5
48.0 kb
32.7 kb
15.3 kb
9.42 kb
6.56 kb
4.36 kb

very similar inserts which were greater than 27 kbs. (It was highly unlikely that the four inserts and one vector fragments obtained upon *Ase* I digestion of the NL1D1 DNA preparation were a result of one NL1D1 clone containing three *Ase* I sites in the insert for the reason that *Ase* I recognition sites (ATTAAT) are very rare in the *Streptomyces* genome which contains 61 to 79% GCs (Wright and Bibb, 1992) and *Ase* I fragments would be too large for packaging by lambda phage heads. Comparisons of NL1D1 and p53 samples showed that the p53 insert corresponded to the largest insert in NL1D1.

When *Ase* I digested NL1D1 and p53 DNA were hybridized with the *bli* probe (Figure 16 B), hybridizing bands larger than 27 kbs were obtained in the lanes containing NL1D1 DNA digests while none was seen in the p53 digests. It could not be determined, however, which of the two bands in the dimer was hybridizing to the *bli* probe due to the limits of resolution. The absence of *bli* hybridizing bands in the p53 DNA showed that there were sequences present in the NL1D1 insert that were missing in the p53 insert although it was difficult to detect any size differences between the p53 insert and the NL1D1 insert by conventional agarose gel electrophoresis. Restriction mapping of the cosmid p53 insert (Figure 8, Section II.12.2) by A. Wong and A. Paradkar showed that it was approximately 45 kbs which is near the packaging limits of lambda and underscores the idea that the insert in NL1D1 could not be too much larger than the p53 insert as otherwise it would not be packaged.

Although *bli* did not hybridize to any of the smaller NL1D1 inserts or to the single p53 insert, it did hybridize to the pFD666 vector fragment. A possible explanation for this observation is that sheared *E. coli* pUC fragments had copurified with the *bli* probe. Similar sequences between the contaminating pUC fragments and vector sequences of pFD666 would allow for hybridization. However, it is more likely that there is similarity betweeen the *bli* probe and pFD666 plasmid sequences.

IV.3 MAPPING THE LOCATION OF THE *BLI* GENE WITH RESPECT TO THE END FRAGMENTS OF P53.

Since the 7 and 9.5 kb *Sac* I fragments designated as the *cas1* region on the p53 restriction map (Figure 8 A, Section II.12.2) were believed to be contiguous sequences (R. H. Mosher, C. Anders and S. E. Jensen, unpublished work), the rearrangement and deletion events at the *bli* locus would have been at the left or right ends of NL1D1 or in an internal region to the right or to the left of the *cas1* region. If deletions took place at the ends, NL1D1 and p53 would have different end sequences and the *bli* gene would be located on sequences just beyond one end of cosmid p53. However, if the deletions that resulted in the loss of sequences containing the *bli* gene were internal to the NL1D1 cosmid, the ends of cosmid p53 and NL1D1 would be similar. These two possibilities were experimentally addressed in two ways. The first approach was an attempt to determine which end of the p53 insert the *bli* gene is linked to by probing genomic DNA digests with *bli* and the right and the left end fragments of the p53 insert. The presence of a common band hybridizing to *bli* and to one of the end fragments of the p53 insert would show the linkage of *bli* with that end. The second approach was to determine whether the ends of the insert in p53 and that of NL1D1 were similar and if so, it would then be concluded that *bli* is present in an internal fragment that was deleted in generating p53. Four probes were used in these experiments. The *bli* probe is a 840 basepair *Cla* I/*Kpn* I fragment from pBIP containing the *bli* gene. The p53 probes consisted of L-4 , the 4 kb *Bgl* II/*Xba* I left end fragment of the p53 insert, R-3.3, the 3.3 kb *Bam* HI/*Hind* III right end fragment of the p53 insert and Bgl-1.6, a 1.6 kb *Bgl* II internal fragment in p53 that lies in the area right of the *cas1* region. (The locations of these probes are shown in Figure 8, A and B, Section II.12.2.)

IV.3.1 Southern Hybridization of Genomic DNA with the *Bli* Gene and the Left and Right Arm of the Insert in p53.

Southern hybridization experiments in which genomic DNA digests were probed with the ends of the p53 insert and the *bli* gene were done to determine whether the *bli* gene was located beyond either end of p53. The probes used were L-4, R-3.3 and the *bli* probe. Genomic DNA samples were doubly digested with *Bgl* II/*Cla* I, *Sac* I/*Cla* I, *Xho* I/*Cla* I, *Bgl* II/*Sac* I and *Bgl* II/*Xho* I and separated on a 0.5% agarose gel (Figure 17). The separated DNA fragments were then transferred to nylon membrane and the membrane hybridized separately with the *bli* probe, R-3.3 and L-4 (Figure 18).

Bgl II/*Cla* I was chosen because the *bli* gene was on a ~ 9kb *Bgl* II/*Cla* I fragment in pBIP, Figure 8 B. The *Sac* I was chosen because it digested p53 into five fragments of different sizes. *Xho* I digestion generated fragments of *S. clavuligerus* genomic DNA that were too large for conventional gel electrophoresis. However, in double digests with the above three enzymes, fragments in the size ranges of 20 kbs and less were

Figure 17. Agarose gel electrophoresis analysis of *S. clavuligerus* genomic DNA double digests. Genomic DNA preparations were doubly digested with 1: *Bgl* II/*Cla* I (lanes 1), 2: *Bgl* II/*Sac* I (lanes 2), 3: *Bgl* II/*Xho* I (lanes 3), 4: *Cla* I/*Sac* I (lanes 4) and 5: *Xho* I/*Cla* I (lanes 5). Triplicate sets of samples were separated on a 0.5 % agarose gel, stained with ethidium bromide, photographed and the image is shown. The lambda molecular size markers are lambda DNA digested with *Pst* I. The separated DNA samples were then transferred onto a nylon membrane for Southern hybridization analysis.

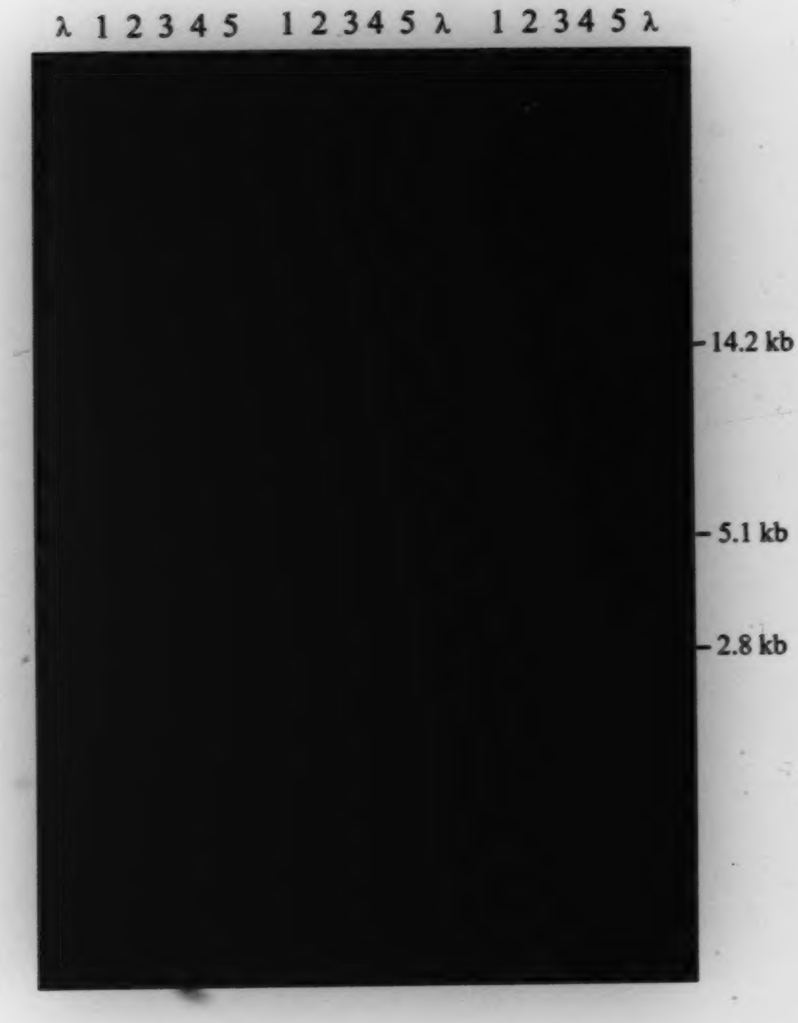
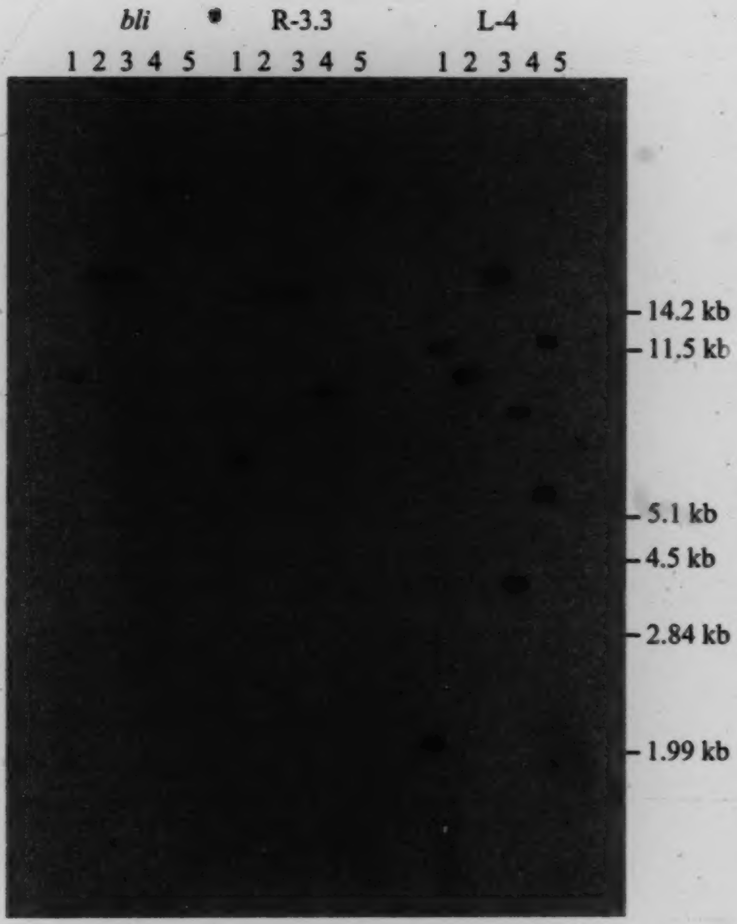

λ 1 2 3 4 5 1 2 3 4 5 λ 1 2 3 4 5 λ
14.2 kb
5.1 kb
2.8 kb

Figure 18. Southern hybridization of genomic DNA digests with the *bli* gene and the left and right end of cosmid p53. Genomic DNA doubly digested and separated on a 0.5 % agarose gel (Figure 17) was blotted onto nylon membrane. The membrane was cut into three sections and each section was then probed with one of three probes: the *bli* probe, a 840 basepair *Cla* I/*Kpn* I fragment containing the *bli* gene ; the L-4 probe, a 4 kilobase fragment corresponding to the left end of the insert in cosmid p53 obtained from *Bgl* II/*Xba* I digestion of p53; and the R-3.3 probe, a 3.3 kilobase fragment corresponding to the right end of cosmid p53 obtained from digestion with *Bam* HI/*Hind* III. The various double digests were: lanes 1, *Bgl* II/*Cla* I; lanes 2, *Bgl* II/*Sac* I; lanes 3, *Bgl II*/*Xho* I; lanes 4, *Cla* I/*Sac* I and lanes 5, *Xho* I/*Cla* I.


bli
R-3.3
L-4
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
14.2 kb
11.5 kb
5.1 kb
4.5 kb
2.84 kb
1.99 kb

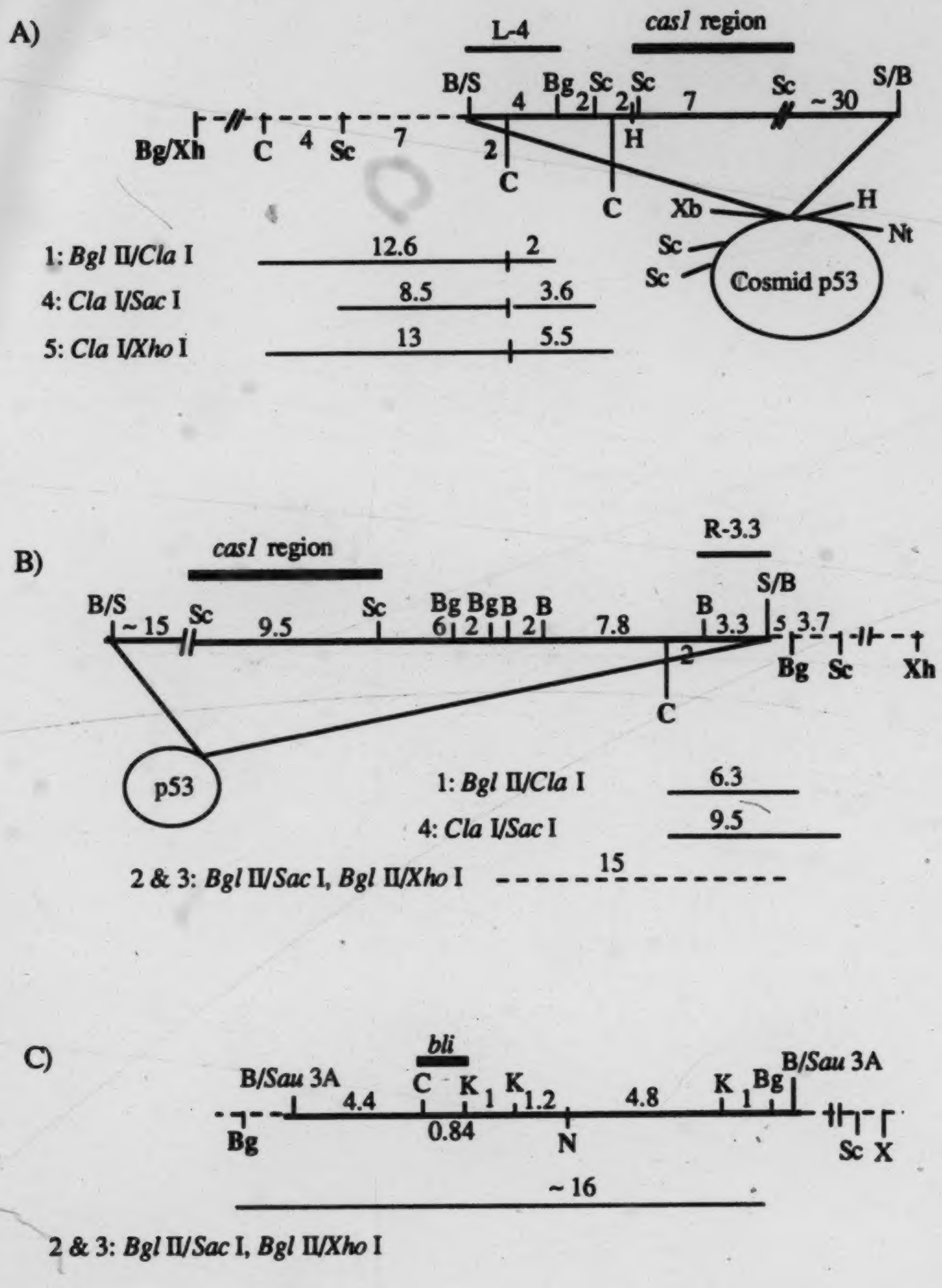
obtained. This experiment was designed to detect a band of less than 15 kb that would hybridize with *bli* and one of the two ends of the insert in p53 thereby proving that the *bli* gene was linked to that particular end of the insert in cosmid p53.

In Figure 18, the first five lanes corresponded to genomic DNA digests probed with the *bli* probe. The next five lanes were the same five genomic DNA digests probed with R-3.3 and the last five lanes were results obtained when probed with L-4. Results from this experiment showed that no unambiguously common bands were seen between genomic DNA digests hybridized with *bli* and either the left or right end probes. Nonetheless, the data generated allowed more mapping information to be derived for the regions beyond the ends of the p53 cosmid insert. The extended map also made it possible to define the minimum distance that *bli* would be beyond the ends of the p53 cosmid insert. Details pertaining to the determination of the restriction map of the areas around fragments L-4 and R-3.3 in the genome obtained are discussed in the following sections. (See Figures 18 and 19 A, B and C.)

IV.3.1.1 Restriction Mapping Regions Left of p53.

In Figure 18, L-4/lane 1, genomic DNA was digested with *Bgl* II/*Cla* I and probed with L-4. Two hybridizing bands were seen, a 2.2 and a 12.6 kb band. This suggested that a *Cla* I site existed within the L-4 probe since no *Bgl* II site existed there (Figure 19 A). The 2.2 kb band corresponded to the portion of L-4 that is on p53 while the 12.6 kb band suggested a *Bgl* II or a *Cla* I site in the genome that is 11.8 kbs upstream of the L-4 end of p53. Figure 18, L-4/lane 4, shows that when genomic DNA was digested with *Cla* I/*Sac* I and probed with L-4, two hybridizing bands of 8.5 and 3.6 kbs were obtained. The presence of two bands in a *Cla* I/*Sac* I digest confirmed the presence of a *Cla* I site in L-4. The smaller 3.6 kb band corresponded to the *Sac* I/*Cla* I fragment on p53 while the larger 8.5 kb fragment showed that a *Sac* I site existed 7 kb upstream of L-4. (The presence of a 12.6 kb fragment in the *Bgl* II/*Cla* I digest, L-4/lane 1, eliminated the possibilility that the site 7 kb upstream of L-4 was a *Cla* I site.) And finally, the two hybridizing bands in DNA digested with *Cla* I/*Xho* I, 13 and 5.5 kb bands, seen in L-4/lane 5 also confirmed the existence of the *Cla* I site in L4 and suggested either the presence of two *Xho* I or two additional *Cla* I sites, one internal to p53 and one beyond the L-4 region. It was likely that the 13 kb fragment was actually a *Cla* I fragment and if so the uncertain *Cla* I or *Bgl* II site 11 kb upstream of L-4 (Figure 18, L-4/lane 1) would then be a *Cla* I site. Although the 5.5 kb fragment could result

Figure 19. Restriction map of genomic sequences beyond the ends of cosmid p53 and beyond the pBIP insert. Extended restriction maps of the regions in the genome that are left (A) and right (B) of the insert in cosmid p53 and beyond the insert in pBIP (C) were obtained from Southern hybridization results from Figure 18. Sequences belonging to the insert in cosmid p53 are indicated by solid lines while dotted lines indicate genomic DNA sequences. Restriction sites and distances between each site are indicated. Restriction sites are given as follows: B, *Bam* HI; Bg, *Bgl* II; C, *Cla* I; H, *Hind* III; Nt, *Not* I; P, *Pst* I; Sc, *Sac* I; S, *Sal* I; X, *Xho* I and Xb, *Xba* I. L-4 and R-3.3 mark the region within cosmid p53 that were used as probes in Southern hybridization experiments. The *cas1* region indicated by a dark line above the 7 kilobase *Sac* I fragment in A) and the 9.5 *Sac* I fragment in B) contains clavam biosynthetic genes and has been shown to be intact and representative of the same area in the genome (R. H. Mosher and C. Anders, unpublished results). Fragments obtained by restriction digestion of genomic DNA that hybridized to L-4, R-3.3 and the *bli* probe are indicated below the region of overlap in in each sequence while numeric values referred to the corresponding lanes in Figure 18 and the type of enzymatic digestion. C). Restriction map of the region in the vicinity of the pBIP insert as determined by hybridization of genomic DNA with the *bli* gene (Figure 18).


A)
L-4
casI region
B/S
Bg
Sc
Sc
Sc
S/B
4
2
2
7
~ 30
Bg/Xh
C
4
Sc
7
2
C
H
C
Xb
H
Nt
Sc
Sc
Cosmid p53
1: *Bgl* II/*Cla* I
12.6
2
4: *Cla* I/*Sac* I
8.5
3.6
5: *Cla* I/*Xho* I
13
5.5
B)
casI region
R-3.3
S/B
B/S
~ 15
Sc
9.5
Sc
Bg Bg B B
6
2
2
7.8
B
3.3
.5
3.7
2
Bg
Sc
Xh
C
p53
1: *Bgl* II/*Cla* I
6.3
4: *Cla* I/*Sac* I
9.5
2 & 3: *Bgl* II/*Sac* I, *Bgl* II/*Xho* I
15
C)
bli
B/*Sau* 3A
C
K
K
K
Bg
B/*Sau* 3A
4.4
1
1.2
4.8
1
Bg
0.84
N
Sc
X
~ 16
2 & 3: *Bgl* II/*Sac* I, *Bgl* II/*Xho* I

from an internal *Xho* I or a second *Cla* I site in p53 it was more likely due to the presence of an internal *Cla* I site as *Cla* I sites (ATCGAT) are more prevalent than *Xho* I sites (CTCGAG) as was seen in genomic DNA that was digested with *Cla* I or *Xho* I (results not shown). In L-4/lane 3, one fragment greater than 17 kb in size was seen when *Bgl* II/*Xho* I digested genomic DNA was probed with L-4 and this suggested a distant *Bgl* II or *Xho* I site upstream of L-4. Figure 19 showed restriction sites determined from mapping cosmid p53 (A. Wong, A. Paradkar and S. E. Jensen, unpublished work) as well as sites determined from genomic hybridization studies discussed here. Sites determined from the genomic hybridization experiments reported here are shown in bold.

IV.3.1.2 Restriction Mapping Regions Right of p53.

When genomic DNA was digested with *Cla* I/*Sac* I and hybridized with R-3.3, a fragment of 9.5 kb was seen, Figure 18, R-3.3/lane 4. This suggested the presence of a *Cla* I site within the insert upstream of the R-3.3 region (Figure 19 B) and a *Sac* I or *Cla* I site downstream of the right end of p53. In Figure 18, R-3.3/lane 1, *Bgl* II/*Cla* I digests showed a band of 6.3 kb when probed with R-3.3. This confirmed the presence of the *Cla* I site upstream of R-3.3 and showed that a *Bgl* II site exists downstream of R-3.3. (The 9.5 kb fragment seen with *Cla* I/*Sac* I digestion eliminated the possibility of this being a *Cla* I site.) In R-3.3/lane 2, the 15 kb fragment in the *Bgl* II/*Sac* I digest was similar to the fragment seen in the *Bgl* II/*Xho* I digest, R-3.3/lane 3, indicating that R-3.3 is on a 15 kb *Bgl* II fragment. This 15 kb *Bgl* II fragment is shown as a dotted line in Figure 19 for the reason that subsequent experiments showed that in the p53 insert, the region to the right of the *cas1* area has undergone rearrangement(s) and deletion(s). For this reason, although it can be concluded that R-3.3 is near one end of a 15 kb *Bgl* II fragment, the actual location of the other *Bgl* II site that should lie within the p53 insert cannot be determined with respect to the p53 restriction map that was determined by A. Wong and A. Paradkar. The largest hybridizing band on the blot was seen in the *Cla* I/*Xho* I digest (R-3.3/lane 5), and this band confirmed that the hybridizing bands seen in R-3.3/lanes 2 and 3 were *Bgl* II fragments. It also confirmed that the potential *Sac* I or *Cla* I site that gave rise to the 9.5 kb band seen in R-3.3/lane 4 was a *Sac* I site and that a *Xho* I or *Cla* I site existed far downstream of the R-3.3 end of p53.

IV.3.1.3 Additional Restriction Mapping of the BIP Cosmid

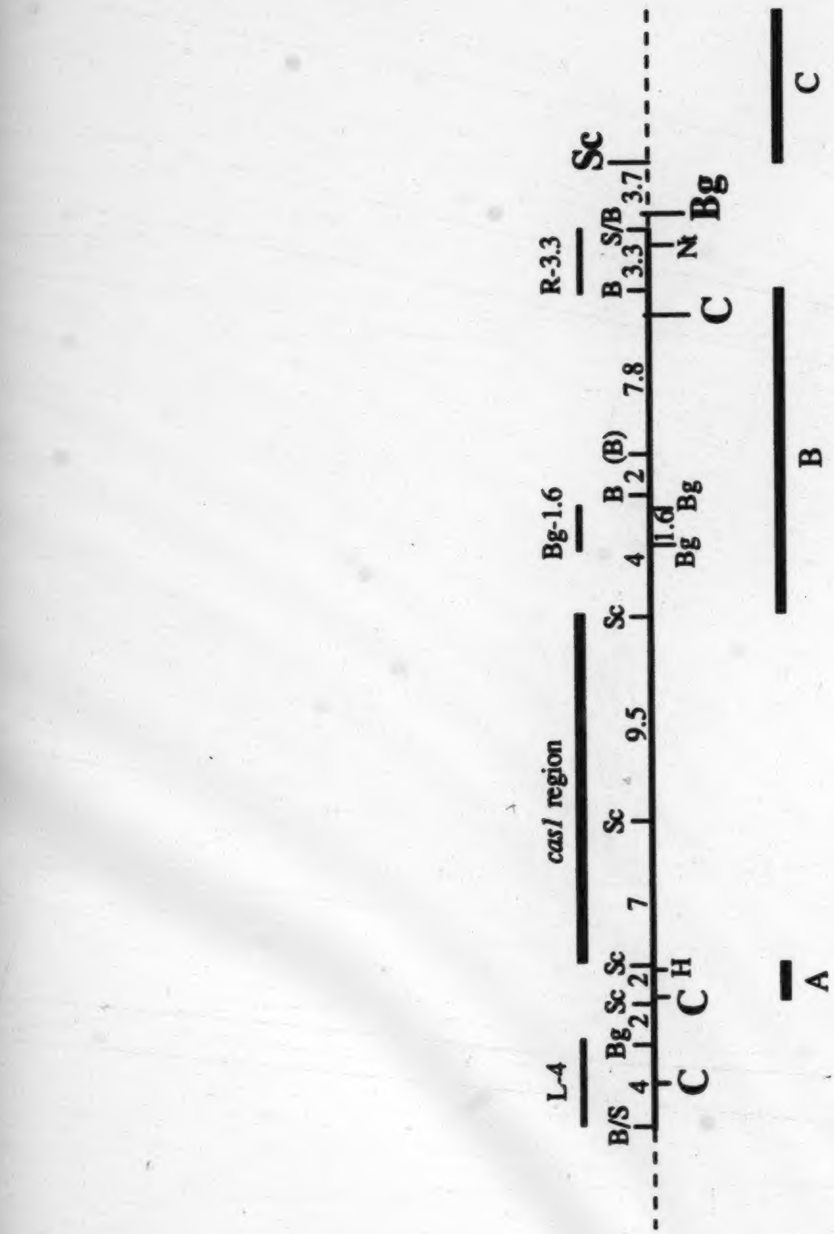
The expected 9.5 kb *Cla* I/*Bgl* II fragment was seen when genomic DNA was digested with *Cla* I/*Bgl* II and probed with the *bli* probe, Figure 18, bli/lane 1. The 16 kb band seen in *Bgl* II/*Sac* I and *Bgl* II/*Xho* I digests, bli/lanes 2 and 3, suggested that the *bli* gene was on a 16 kb *Bgl* II fragment (Figure 19 C). It cannot be concluded that this large *Bgl* II fragment is not the same as the two hybridizing bands seen when *Bgl* II/*Sac* I and *Bgl* II/*Xho* I double digests were probed with R-3.3 (R-3.3/lanes 2 and 3) as the blots that were probed with the *bli* gene and with R-3.3 were two separate blots obtained from two separate gels. If the large 16 kb *Bgl* II fragment on which *bli* is located also contains R-3.3, then the *bli* locus is linked to the right end of the p53 insert and would then be right of the *cas1* region (Figure 20 A). The large bands seen in *Cla* I/*Sac* I and *Cla* I/*Xho* I digested DNA, *bli*/lanes 4 and 5, suggested that there was a *Sac* I and a further *Xho* I site in genomic DNA in regions far downstream of sequences on the cloned insert in pBIP.

These genetic mapping results show that in the genome, the *bli* gene is not within 11 kbs beyond the left end of L-4 nor is it on the 5.5 kb *Cla* I fragment spanning part of L-4 (Figure 19 A) since there were no common hybridizing band between blots probed with the *bli* probe and L-4. The absence of a common hybridizing band in *Cla* I/*Sac* I digests probed with the *bli* probe and R-3.3 showed that *bli* cannot be within a 9.5 kb fragment defined by the *Cla* I and *Sac* I sites flanking R-3.3 (Figure 19 B). It is possible that the site of the *bli* deletion is located on a 2 kb *Sac* I fragment immediately left of the 7 kb part of the *cas1* region (Figure 20 A) or that it is located on sequences between the *Sac* I site that defines the right end of the *cas1* region and a *Cla* I site 2 kbs to the left of R-3.3 (Figure 20 B) both of which could have been rearranged or could have undergone deletions resulting in the loss of the *bli* gene in p53. A third possibility is that *bli* is located further than 4 kb beyond R-3.3 (Figure 20 C) and that the absence of *bli* in p53 resulted from deletions in NL1D1 of sequences right of R-3.3.

IV.3.2 Southern Hybridization of the Clavam Cosmids NL1D1 and p53 and the *Bli* Cosmid pBIP with L-4, R-3.3 and the 840 base pair *bli* probe.

To determine if the ends of cosmid p53 and NL1D1 were similar, Southern hybridization of cosmid p53 and NL1D1 was done. The *bli* containing cosmid pBIP was included for comparison with NL1D1. The three cosmids were digested with *Bgl*

Figure 20. Possible locations of the pBIP insert within the p53 fragment based on genomic hybridization experiments. Solid lines in the diagram indicate cosmid insert sequences while dotted lines indicate regions determined from genomic Southern hybridization experiments. New restriction sites determined from genomic Southern hybridization experiments are shown in larger bold font. A) The pBIP insert could be in the small *Sac* I fragment that is left of the p53 insert. The pBIP insert could also be located in the region right of the *cas1* area either internal to the existing p53 insert (B) or beyond the right end of the p53 insert (C).



B/S
4
L-4
C
Bg
2
Sc
C
2
Sc
H
A
7
cas1 region
Sc
9.5
Sc
4
Bg-1.6
Bg
1.6
Bg
B
2
(B)
B
7.8
C
B
3.3
R-3.3
N
S/B
Bg
3.7
Sc
C

II/*Cla I*, *Hind* III, *Nco* I and *Sac* I. The *Bgl* II/*Cla* I double digest was done to determine if the 9 kb fragment containing the *bli* gene in pBIP was also present in NL1D1. The *Nco* I digest was included as a positive control since the *bli* gene was shown to be present on a 5 kb *Nco* I fragment in NL1D1 (section IV.2). The digested DNA preparations were separated on a 0.5 % agarose gel and blotted for Southern hybridization. The probes used were the 840 basepair *bli* probe and the L-4, R-3.3 and Bgl-1.6 fragments of the insert in p53. Figure 21 shows an ethidium bromide stained agarose gel of p53, NL1D1 and pBIP digested DNA samples prior to Southern blotting. Multiple bands in the NL1D1 digests are due to the various insert fragments in the NL1D1 cosmid preparation while the fainter bands seen in the p53 digests indicate that even p53, the 'stable derivative of NL1D1, is undergoing rearrangements. Figures 22, 23, and 24 are results obtained from Southern hybridization experiments with the probes L-4, R-3.3 and the 840 basepair *bli* probe.with *Bgl* II/*Cla* I, *Hind* III, *Nco* I and *Sac* I, the next four lanes correspond to NL1D1 similarly digested and the last four lanes correspond to pBIP also digested with the same enzymes. In Figures 22 and 23, the blots were probed with R-3.3 and L-4 respectively while in Figure 24, the blot was probed with the 840 base pair *bli* probe.

Comparisons of Figures 22, 23 and 24 revealed several qualitative differences. First, in Figure 22 and 23, the absence of hybridizing bands in the pBIP lanes indicated that neither the left (L-4) nor the right (R-3.3) region of the p53 insert is present on the pBIP cosmid. Secondly, in Figure 24, the absence of hybridizing bands in the p53 lanes when the blot was probed with the 840 basepair *bli* probe confirmed the PCR result that p53 does not contain the *bli* gene. Third, Figure 24 shows that when probed with the *bli* probe, hybridizing bands in the NL1D1 lanes were significantly fainter than the hybridizing bands in the pBIP lanes suggesting that intact NL1D1 cosmids carrying inserts containing the *bli* gene comprised only a small proportion of the total since similar amounts of cosmid DNA samples were loaded on the gel as shown in Figure 21. This suggests that the NL1D1 preparation was a mixed population of cosmids, few of which still retained the intact *bli* locus while most contained deleted and rearranged derivatives. And last, when hybridized with R-3.3 or L-4 (Figure 22 and 23), striking similarity in the banding patterns of the p53 and NL1D1 DNA was seen. This suggested that deletions that gave rise to p53 occurred in an internal region such that the ends of p53 and NL1D1 were similar. Fainter hybridizing bands in both the p53 and

Figure 21. Agarose gel electrophoresis analysis of various digests of p53, NL1D1 and pBIP. Cosmid preparations of p53, NL1D1 and pBIP were digested with *Bgl* II/*Cla* I (lanes 1), *Hind* III (lanes 2), *Nco* I (lanes 3) and *Sac* I (lanes 4) and separated on a 0.5 % agarose gel. Lanes containing DNA size standards are labeled as λ and consisted of λ DNA digested with *Pst* I.

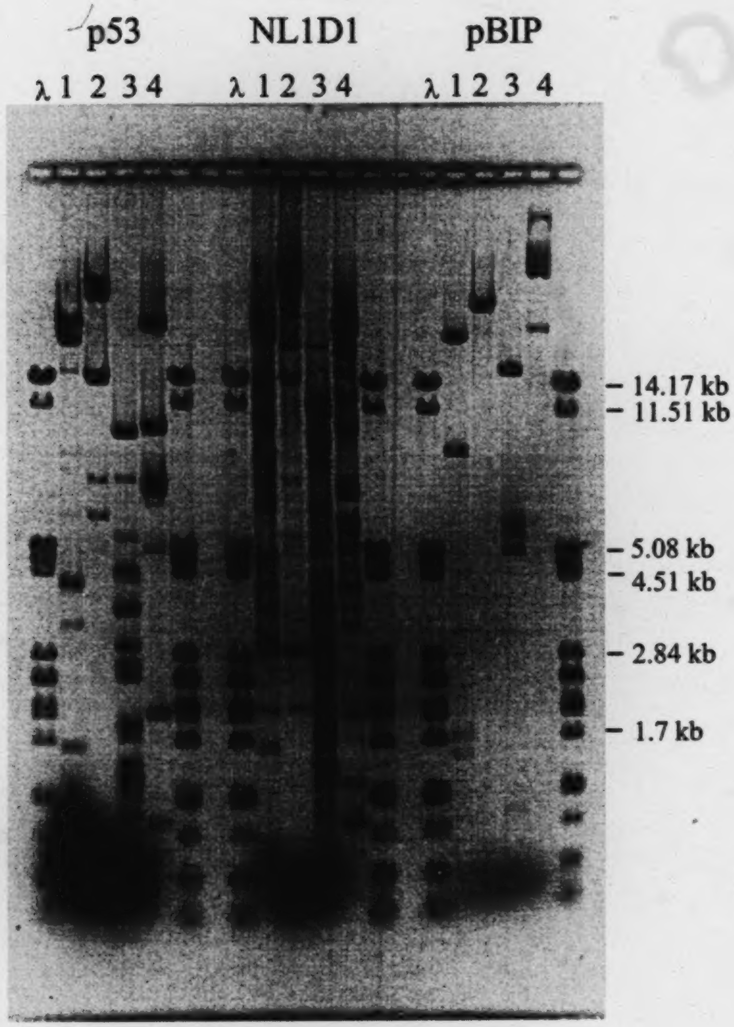

p53
NL1D1
pBIP
λ 1 2 3 4
λ 1 2 3 4
λ 1 2 3 4
14.17 kb
11.51 kb
5.08 kb
4.51 kb
2.84 kb
1.7 kb

Figure 22. Southern hybridization of cosmid p53, NL1D1 and pBIP with R-3.3. Cosmids p53, NL1D1 and pBIP were doubly digested with *Bgl* II/*Cla* I (lanes 1), *Hind* III (lanes 2), *Nco* I (lanes 3) and *Sac* I (lanes 4) and then separated on a 0.5 % agarose gel. The gel was blotted onto nylon membrane and the membrane probed with α^{32}P-dCTP labelled R-3.3, the right end fragment of p53 obtained from digestion with *Bam* HI and *Hind* III.

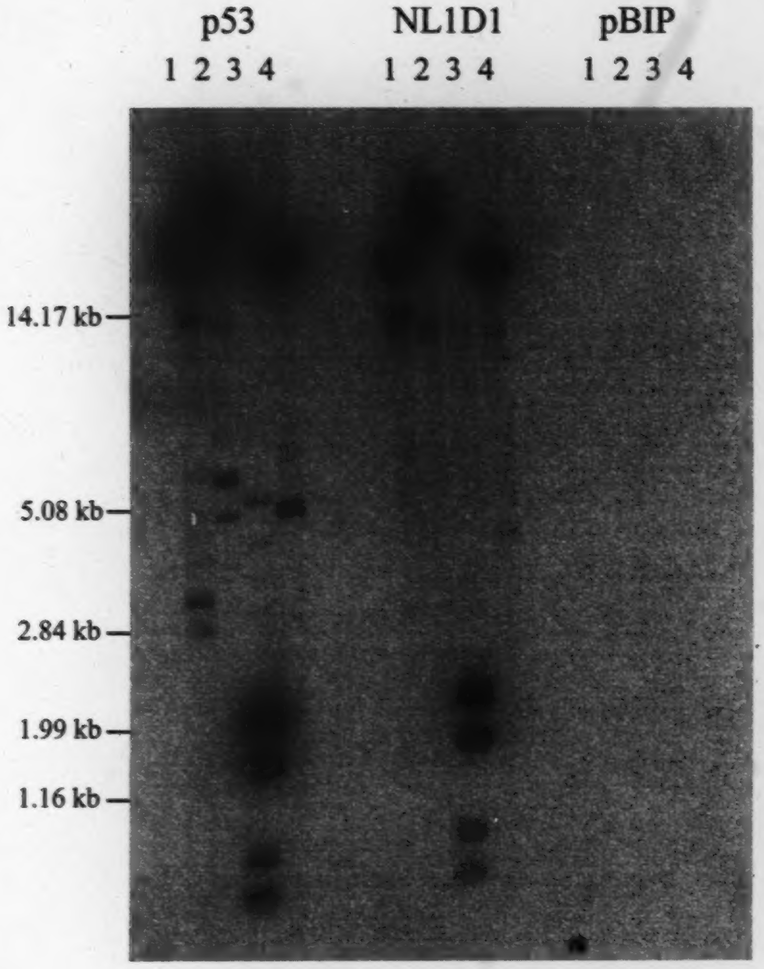

p53
NL1D1
pBIP
1 2 3 4
1 2 3 4
1 2 3 4
14.17 kb
5.08 kb
2.84 kb
1.99 kb
1.16 kb

Figure 23. Southern hybridization of cosmids p53, NL1D1 and pBIP with L-4. Cosmids were doubly digested with *Bgl* II/*Cla* I (lanes 1), *Hind* III (lanes 2), *Nco* I (lanes 3) and *Sac* I (lanes 4) and then separated on a 0.5 % agarose gel. The gel was blotted onto a nylon membrane and the membrane probed with α^{32}P-dCTP labelled L-4, the left end fragment of p53 obtained by digestion with *Bgl* II and *Xba* I.

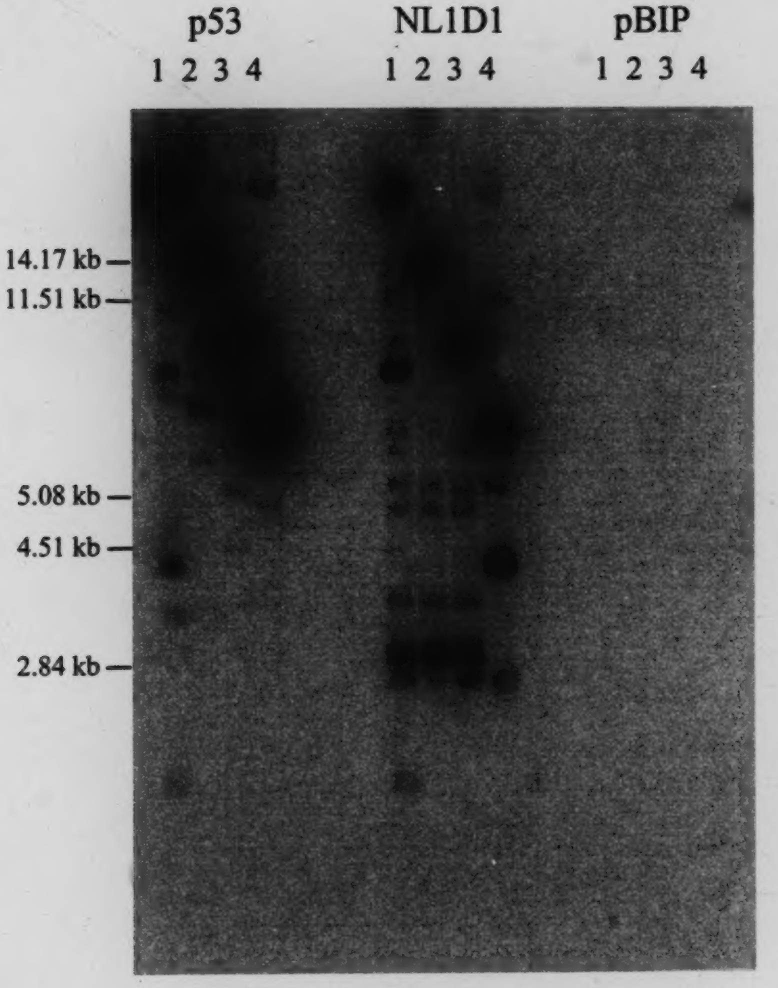
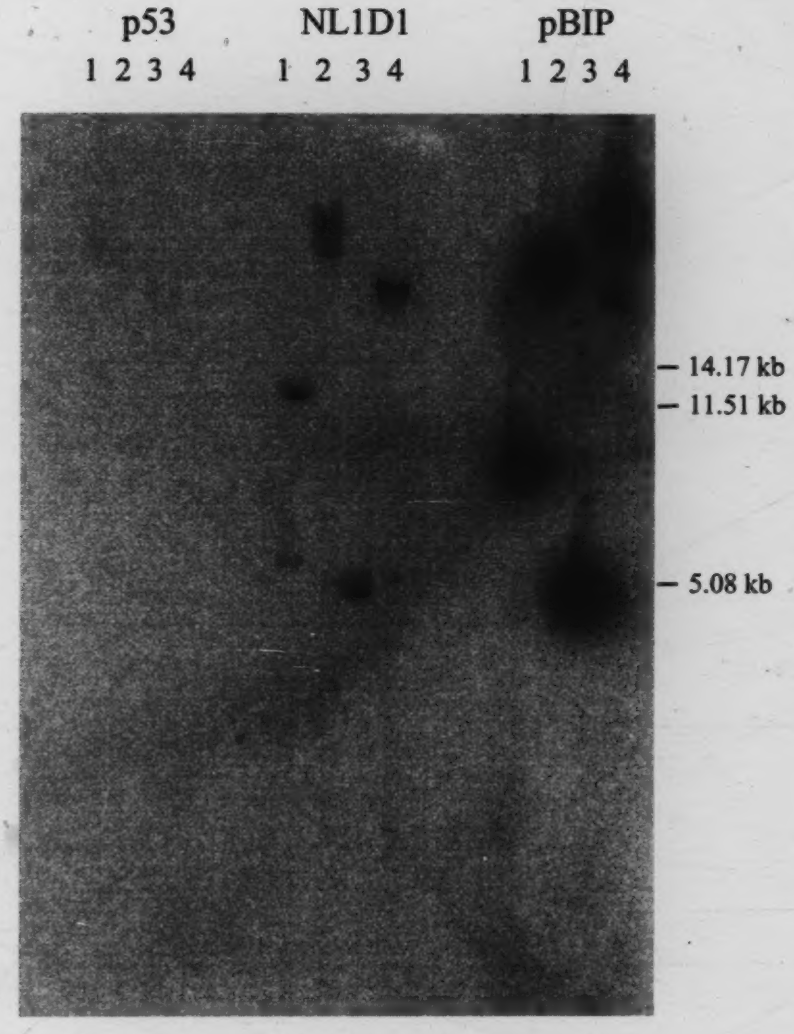

p53
1 2 3 4
NL1D1
1 2 3 4
pBIP
1 2 3 4
14.17 kb
11.51 kb
5.08 kb
4.51 kb
2.84 kb

Figure 24. Southern hybridization of cosmids p53, NL1D1 and pBIP with the *bli* probe. Cosmids were doubly digested with *Bgl* II/*Cla* I (lanes 1), *Hind* III (lanes 2), *Nco* I (lanes 3) and *Sac* I (lanes 4) and then separated on a 0.5 % agarose gel. The gel was blotted onto a nylon membrane and the membrane probed with α^{32}P-dCTP labelled *bli* probe, a 840 basepair *Cla* I/*Kpn* I fragment containing the *bli* gene.


p53
NL1D1
pBIP
1 2 3 4
1 2 3 4
1 2 3 4
14.17 kb
11.51 kb
5.08 kb

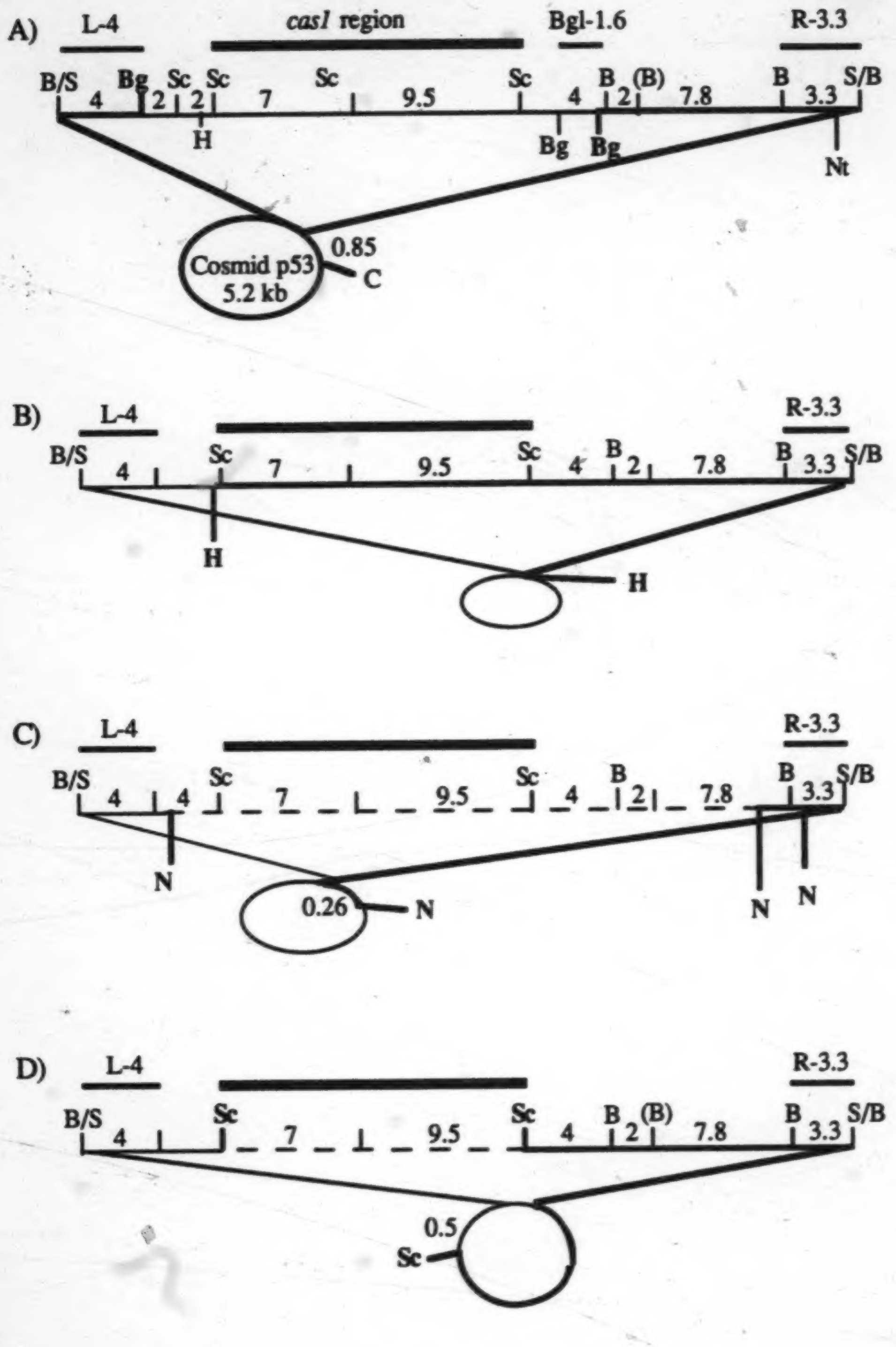
NL1D1 DNA containing lanes of Figures 22 and 23 are presumed to represent rearranged and deleted forms of the two cosmids.

In Figure 22, the *Bgl* II/*Cla* I digests of p53 and NL1D1 (lanes 1) probed with R-3.3 showed a band having an apparent molecular weight greater than 20 kb whereas mapping studies suggested the band should be 14 kbs. This greater than 20 kb band could be the result of an incomplete digestion of p53 and NL1D1 in the *Bgl* II/*Cla* I digest resulting in a *Bgl* II fragment (Figure 25 A) consisting of the left fragment of the insert in p53 (4 kbs), the vector (5.2 kbs) and the right fragment of the insert in p53 (11 kbs). The presence of this same signal in p53 and NL1D1 probed with L-4 (Figure 23, lanes 1) confirmed that both ends of the p53 insert (R-3.3 and L-4) were present on this fragment. From this result, it was concluded that the insert in NL1D1 and p53 had similar end regions.

In the *Hind* III digests of p53 and NL1D1, a hybridizing band that was greater than 30 kb was seen when the digests were probed with R-3.3 (Figure 22, p53 and NL1D1 lanes 2). Since two *Hind* III sites existed in p53, one right of the *cas1* region and the second in the vector immediately left of the insert, this large *Hind* III fragment was believed to be the 36 kb region encompassing most of the insert with the exception of the 8 kb left fragment (Figure 25 B). Again, the large size of this fragment made it difficult to infer if the actual bands in p53 and in NL1D1 were of indentical sizes. A 13 kb band was seen in *Hind* III digests of p53 and NL1D1 when probed with L-4 (Figure 23, lanes 2). This fragment consisted of the left 8.3 kb fragment of the p53 insert and 5.2 kb of the vector. The similarity in size of this fragment in NL1D1 and p53 was the first evidence that the 8.3 kb fragment, left of the *cas1* region in p53 and NL1D1, are the same and suggested that the deletions and rearrangements in the *bli* region were right of the *cas1* region. It was concluded from the results so far that the *bli* locus is likely in the region right of the *cas1* area.

In the *Nco* I digests of p53 and NL1D1 probed with R-3.3 (Figure 22, p53 and NL1D1 lanes 3), two similar bands of 1.2 and 1.7 kbs were seen. Since the vector contains an *Nco* I site 0.26 bp beyond the right end of the insert in p53, it was concluded that two *Nco* I sites exist within 2.5 kb upstream from the right end of the insert (Figure 25 C) and that the right 2.5 kb fragments in p53 and NL1D1 are similar. When probed with L-4 (Figure 23), *Nco* I digests of p53 and NL1D1 both showed a band of 9.5 kbs which was evidence that an *Nco* I site existed 5 kbs from the left end of

Figure 25. Schematic diagram of cosmid p53 and fragments obtained from Southern hybridization of various enzymatic digestions using the probes R-3.3 and L-4. The regions indicated by the different lines mark the different fragments that were obtained upon hybridization of the *Bgl* II/*Cla* I (A), *Hind* III (B), *Nco* I (C) and *Sac* I (D) digested cosmid p53 with the R-3.3 probe (bold lines) and the L-4 probe (regular lines). Restriction enzyme sites are indicated by the following abbreviations: *Bam* HI, B; *Bam* HI/*Sal* I, B/S; *Bgl* II, Bg; *Cla* I, C; *Hind* III, H; *Nco* I, N; *Not* I, Nt and *Sac* II, Sc.


A)
L-4
cas1 region
Bgl-1.6
R-3.3
B/S
Bg
Sc
Sc
Sc
Sc
B
(B)
B
S/B
4
2
2
7
9.5
4
2
7.8
3.3
H
Bg
Bg
Nt
Cosmid p53
5.2 kb
0.85
C
B)
L-4
R-3.3
B/S
Sc
Sc
B
B
S/B
4
7
9.5
4
2
7.8
3.3
H
H
C)
L-4
R-3.3
B/S
Sc
Sc
B
B
S/B
4
4
7
9.5
4
2
7.8
3.3
N
0.26
N
N
N
D)
L-4
R-3.3
B/S
Sc
Sc
B
(B)
B
S/B
4
7
9.5
4
2
7.8
3.3
0.5
Sc

the p53 and NL1D1 insert and further confirmed that the left 5 kbs were similar in p53 and NL1D1.

In the *Sac* I digests of p53 and NL1D1 probed with R-3.3 (Figure 22, lanes 4), again a fragment with an apparent size greater than 20 kb was seen in both cases. This was believed to consist of the 19 kb fragment right of the *cas1* region in the p53 insert and 3.7 kb of vector DNA (Figure 25 D). Again, the large size made it difficult to conclude whether there were differences in the sizes of the bands seen in NL1D1 and p53. In p53 and NL1D1 digests probed with L-4 (Figure 23, lanes 4) however, a band of 7.5 kb was seen in both cases. This band corresponded to the 6 kb left fragments of the p53 insert and a 2 kb fragment from the vector region. Again, the left end of p53 and NL1D1 were shown to be the same.

From these results, it was concluded that the left 25 kbs of the p53 insert which consisted of the *cas1* region and the area left of it, resembled the insert in NL1D1. Furthermore, the deletions and rearrangements that had taken place to generate p53 had occurred between the *Sac* I site that marked the right end of the *cas1* region on the p53 insert and the first *Bgl* II site from the right end of the cosmid (Figure 20 B). This is confirmed by the finding that the *bli* locus is on the larger of the two *Hind* III fragments obtained when p53/NL1D1 was digested with *Hind* III (Figure 25 B).

The *bli* locus in NL1D1 had also undergone deletions and/or rearrangements. When probed with the *bli* probe, *Bgl* II/*Cla* I digested NL1D1 and pBIP DNA showed hybridizing bands of different sizes (Figure 24, NL1D1 and pBIP lanes 1). The 9 kb fragment seen in pBIP was replaced by a 12.5 kb fragment in NL1D1. Since genomic DNA hybridization showed that *bli* is located on an approximately 9 kb fragment (Section IV.3.1.3), the rearrangment/deletion events must have taken placed in the NL1D1 insert. (This conclusion has been confirmed in a later experiment.) Since both *Nco* I digests of pBIP and NL1D1 showed a 5 kb band when probed with the *bli* gene, the rearrangements/deletions in NL1D1 must have occurred outside the 5 kb *Nco* I fragment.

IV.3.3 Southern Hybridization of the Clavam Cosmids NL1D1 and p53 and the *Bli* Cosmid pBIP with the Bgl-1.6 probe.

The results obtained so far indicated that the *bli* gene is located in the region of the NL1D1 insert that is to the right of the *cas1* region. To confirm this conclusion, the

Southern blot that was probed with the *bli* probe (Figure 24) was stripped and rehybridized with a fragment from the region right of the *cas-1* area. The fragment chosen was a 1.6 kb *Bgl* II fragment (Bgl-1.6) shown in Figure 8 (Section II.12.2, Materials and Methods). The autoradiogram in Figure 26 was obtained when the blot that had been probed with the *bli* probe shown in Figure 24 was stripped and reprobed with the Bgl-1.6 probe. In *Bgl* II/*Cla* I digests, (p53 and NL1D1 lanes 1), the bands seen corresponded to probe to probe hybridization. The 4.5 kb band obtained in lanes 3 (*Nco* I digested p53 and NL1D1 DNA) showed that the *bli* gene and the 1.6 kb *Bgl* II fragment were not on the same *Nco* I fragment since no signals were seen in the pBIP DNA samples. Furthermore, the *Nco* I fragment containing the *bli* gene was shown in previous experiments to be 5 kb.

The signals in NL1D1 lanes 2 and 4 which corresponded to *Hind* III and *Sac* I fragments were the same as those seen hybridizing to the *bli* probe (Figure 24, NL1D1 lanes 2 and 4). This result showed the linkage of the *bli* gene with the 1.6 kb *Bgl* II fragment and confirmed that the *bli* locus is in the region to theright of the *cas1* area.

IV.3.4 Southern Analysis of the Region Right of the *Cas1* Area.

To further localize the *bli* gene within the 17 kb region right of the *cas1* area and to show that the insert in NL1D1 or p53 has undergone rearrangement in this area, genomic DNA, NL1D1, p53 and pBIP were digested with *Sac* I/*Cla* I, separated on a 0.8 % agarose gel, DNA fragments transferred to a nylon membrane and hybridized with the Bgl-1.6 probe and the *bli* probe. Results are shown in Figure 27. Comparisons of hybridizing bands in p53, NL1D1 and genomic DNA (Figure 27 A: lanes 2, 3 and 4 respectively) showed that the region in the vicinity of Bgl-1.6 has been rearranged since hybridizing bands obtained were different in all three cases. When hybridized with the *bli* probe (Figure 27 B), p53 DNA showed an unexpected faint hybridizing band upon one week exposure (lane 2). The hybridizing signals in p53, NL1D1 and genomic DNA (lanes 2, 3 and 4 respectively), showed variable sizes indicating that the region spanning the *bli* locus also differed in all three contexts.

From these results, it was concluded that the region to the right of the *cas1* area has undergone deletions and/or rearrangements. The weakly hybridizing *bli* band in p53 could be an indication that a small proportion of the p53 preparation still contains forms of the insert that have the *bli* gene.

Figure 26. Southern hybridization of cosmids p53, NL1D1 and pBIP with Bg-1.6. The blot probed with the *bli* probe in Figure 24 was stripped with boiling 0.1 % SDS and reprobed with α^{32}P-dCTP labelled Bg-1.6, a 1.6 kilobase *BglII* fragment internal to the insert in cosmid p53. Cosmid preparations of p53, NL1D1 and pBIP were digested with *Bgl* II/*Cla* I (lanes 1), *Hind* III (lanes 2), *Nco* I (lanes 3) and *Sac* I (lanes 4) and separated on a 0.5 % agarose gel. Lanes containing DNA size standards are labeled as λ and consisted of λ DNA digested with *Pst* I.

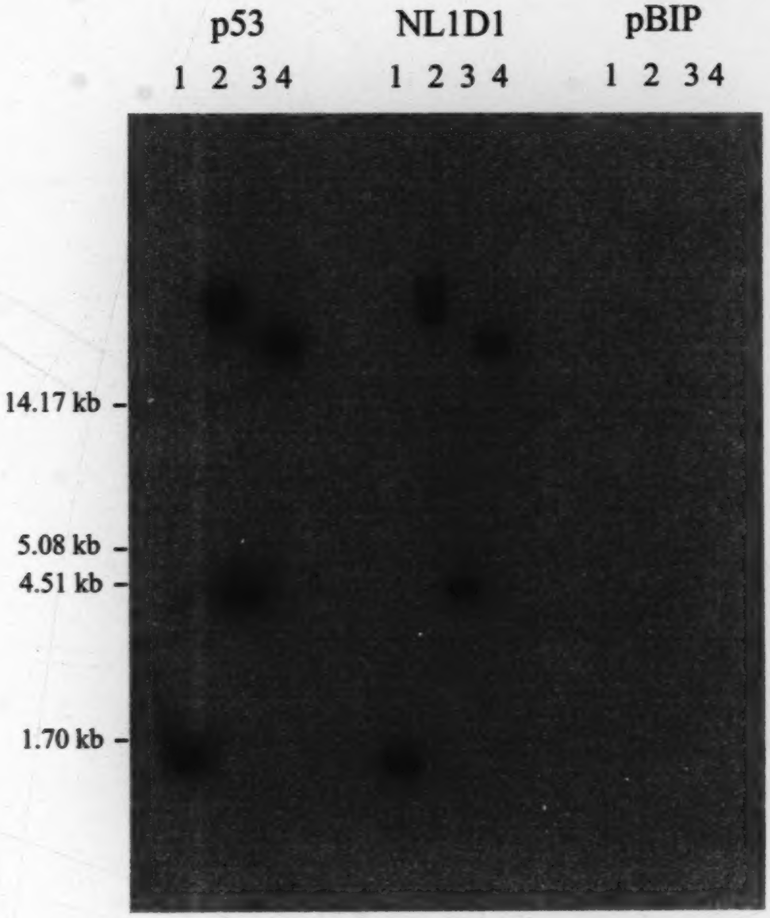
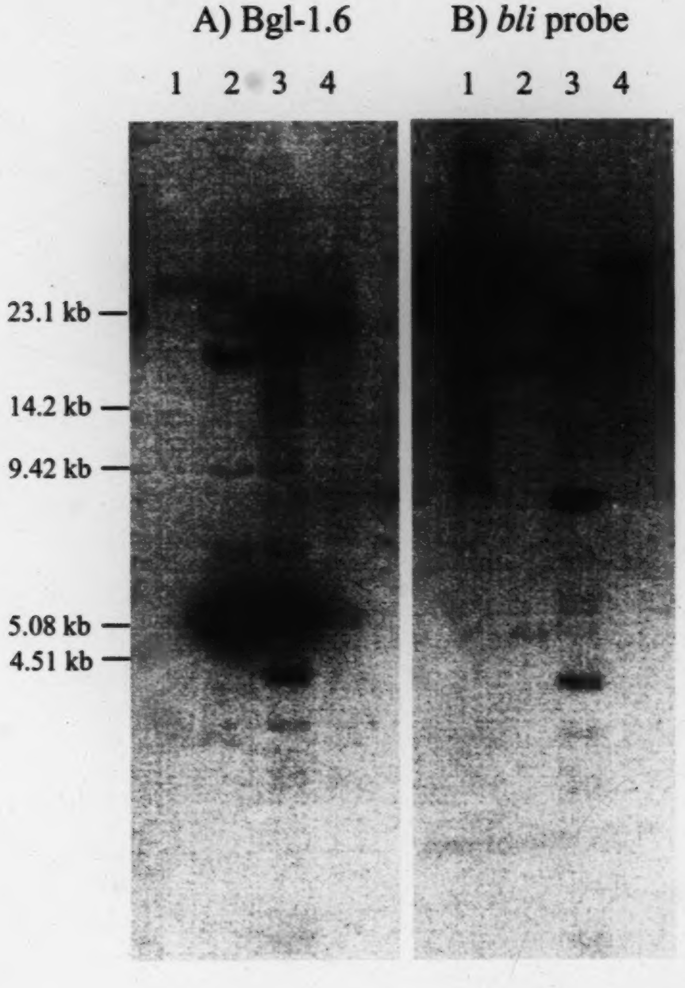

p53
NL1D1
pBIP
1 2 3 4
1 2 3 4
1 2 3 4
14.17 kb
5.08 kb
4.51 kb
1.70 kb

Figure 27. Southern Hybridization of the *Sac* I/*Cla* I digested p53, NL1D1 and genomic DNA with the *bli* probe and the Bgl-1.6 probe. p53, NL1D1 and genomic DNA were digested with *Sac* I/*Cla* I and the digests were separated on a 0.5 % agarose gel. The gel was then transferred onto a nylon membrane and hybridized with the Bgl-1.6 probe (A) and the *bli* probe (B). The membrane was then exposed to X-ray film for 1 week in order to detect hybridizing signals from lanes containing genomic DNA samples. Lanes 1 contained pBIP DNA; lanes 2, p53 DNA, lanes 3, NL1D1 DNA and lanes 4 contained genomic DNA.

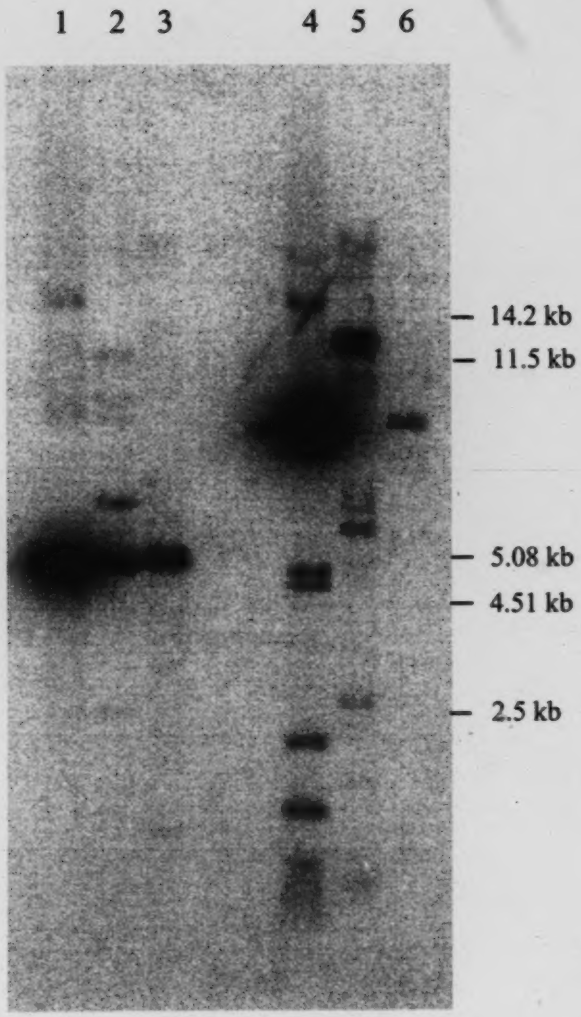

A) Bgl-1.6
B) *bli* probe
1 2 3 4
1 2 3 4
23.1 kb
14.2 kb
9.42 kb
5.08 kb
4.51 kb

IV.3.5 Comparisons of Genomic DNA, NL1D1 and pBIP by Southern Hybridization With the *Bli* Probe.

Since the *bli* locus could not be localized further due to rearrangements and deletions within the p53 and NL1D1 inserts, the next experiment was designed to determine the largest fragment containing the *bli* gene that is conserved in pBIP, NL1D1 and genomic DNA. Since the *bli* gene has been shown to be located on a 5 kb *Nco* I fragment (this study) and on a 9 kb *Bgl* II/ *Cla* I fragment (Doran *et al.*, 1990), genomic DNA, NL1D1 and pBIP DNA were digested with *Nco* I and *Bgl* II/*Cla* I. Figure 28 shows the results obtained when the DNA digests were separated on a 0.5% agarose gel, transferred to a nylon membrane and hybridized with the *bli* probe. Lanes 1, 2 and 3 contained respectively pBIP, NL1D1 and genomic DNA samples digested with *Nco* I, while lanes 4, 5 and 6 contained the same DNA samples digested with *Bgl* II/*Cla* I. Comparisons of lanes 1, 2 and 3 showed that the 5 kb *Nco* I fragment on which the *bli* gene is located could be seen in pBIP (lane 1), NL1D1 (lane 2) and in genomic DNA (lane 3).

Comparisons of lanes 4, 5 and 6 showed that the *Bgl* II/*Cla* I fragment in NL1D1 that hybridizes to the *bli* probe (lane 5) was larger than that seen in pBIP and genomic DNA (lanes 4 and 6 respectively), confirming that in NL1D1, the region beyond the 5 kb *Nco* I fragment containing the *bli* locus had been rearranged. However, in pBIP, the 9 kb *Bgl* II/*Cla* I fragment containing the *bli* gene was representative of the genome as was discussed earlier.

Figure 28. Southern hybridization analysis of the *bli* locus in pBIP, NL1D1 and genomic DNA. pBIP, NL1D1 and genomic DNA were digested with *Nco* I and *Bgl* II/*Cla* I. The digests were separated on a 0.8 % agarose gel, transferred onto nylon membrane and hybridized with the *bli* probe. Lanes 1, 2 and 3 contained pBIP, NL1D1 and genomic DNA digested with *Nco* I respectively while lanes 4, 5 and 6 contained the same DNA samples digested with *Bgl* II/*Cla* I.


1
2
3
4
5
6
14.2 kb
11.5 kb
5.08 kb
4.51 kb
2.5 kb

V. RESULTS: SEQUENCE ANALYSIS

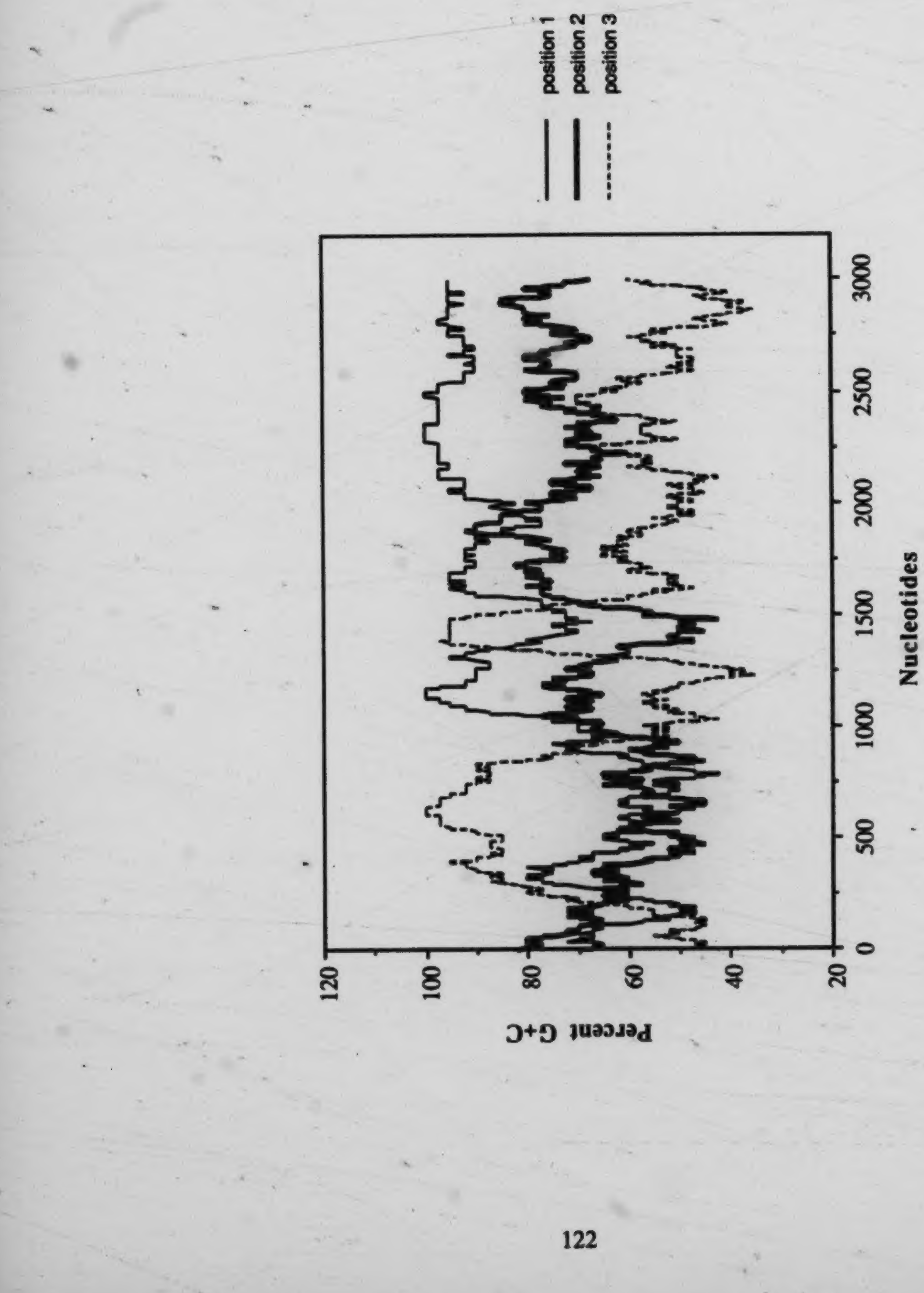
Homologs of clavulanic acid biosynthetic genes, in addition to the *cas1/cas2* genes, are believed to exist in *S. clavuligerus* (S.E. Jensen, personal communication). Since *bli* and the *cdo1* (*cas1 downstream orf-1*) gene in the *cas1* cluster are similar to *blp* and *cefG*, respectively, in the cephamycin cluster, it was hoped that sequencing the area around *bli* might reveal additional homologs of the β-lactam genes. Furthermore, identification of open reading frames in the vicinity of the *bli* gene would be useful for determining the functional context of BLIP. For these reasons, the regions upstream and downstream of the *bli* gene were sequenced from the pBIP cosmid using custom designed oligonucleotide primers. The direction and sequence of the primers are summarized in Section II.13.2.

Results showed that despite similarities between *blp* and *bli*, the genes surrounding *bli* did not resemble those surrounding *blp*. A. Paradkar identified an ORF, *bliup1*, upstream and divergent to the *bli* gene (unpublished work). *bliup1* did not show homology to any known genes. A region consisting of approximately 1 kb beyond this *bliup1* gene was sequenced and no ORFs were detected.

The area downstream of the *bli* gene was also sequenced. Several ORFs were identified in a two kb region immediately downstream of the *bli* gene. Results of frame analysis of a 3127 nucleotide segment consisting of the *bli* gene and the 2 kb region downstream are shown in Figure 29. The percent of Gs and Cs was determined for a window of 120 nucleotides along the 3 kb region. Each of the three plots in Figure 29 represents a position in the first three bases of the sequence. Position 1 is the first base, position 2, the second and position 3, the third base of the sequence. In the frame analysis graph, each of the three curves corresponds to each of the three positions in a codon. As the *Streptomyces* genome contains 61-79 % GC (Wright and Bibb, 1992), the wobble position of *Streptomyces* genes generally shows percent GC in the range of 76-97 %. Therefore, the curve displaying the highest percent GC represents the wobble position of a codon in *Streptomyces* genes while the curve showing the lowest percent GC represents the middle base of a codon. The first position of a codon usually has intermediate percent GC. Regions in the plots where the three lines meet indicate the ends of open reading frames or regions of frame shifts. The first 1 kb in the plot shows the *bli* ORF.

Frame analysis of nucleotides 1000-1600 identified two very small ORFs. These two ORFs were assigned the names *atp-A*, (ABC-transporter protein-Walker A) and

Figure 29. Frame analysis of the *bli* gene and the 2 kb sequence downstream of the *bli* gene by percent GC determination. The percent GC is determined for a 120 base pair window along the length of the sequence. Position 1 is the first nucleotide of the sequence; position 2 the second and position 3 the third nucleotide of the sequence being analysed.


position 1
position 2
position 3
Percent G+C
120
100
80
60
40
20
0
500
1000
1500
2000
2500
3000
Nucleotides

atp-B (ABC-transporter protein-Walker B) since NCBI/BLASTx analysis showed that both ORFs have high homology to ABC transporter proteins from both Gram positive and Gram negative organisms.

ABC transporter proteins are found in prokaryotic and eukaryotic organisms and are known to be involved in importing oligopeptides, amino acids, sugars and inorganic ions as well as the export of capsule polysaccharides, antibiotics, hydrophobic drugs and proteases lacking signal peptides (Higgins, 1992). ABC transporters use the energy obtained from the hydrolysis of ATP to transport solutes across cell membranes. They usually consist of two highly hydrophobic membrane-spanning domains, (MSDs), each having six membrane-spanning segments. Two MSDs form a hydrophilic pore through which solutes pass. MSDs are believed to determine substrate specificity. Little sequence conservation exists among the MSDs so far characterized which is not surprising considering the enormous variety of substrates transported.

The ATP-binding domains, peripherally attached to the periplasmic side of the cell membrane, bind and hydrolyse ATP. Interactions between the ATP-binding domains and the MSDs allow for coupling the energy of ATP hydrolysis to transport. The ATP-binding domains of these proteins are highly conserved, each having two motifs, the Walker A and B motifs. These form the ATP-binding pocket found in all such proteins. The consensus sequences for the Walker A and B sites are G-X-G-K-S-T and D-D/E/S-P/A respectively (Mendez and Salas, 1998). The ATP-binding domain is, in general, about 200 amino acids and can exist alone or fused to second ATP-binding domain or a MSD.

Figure 30 shows the nucleotide sequence of the 2.4 kilobase region containing the 3' part of the *bli* gene and the region downstream of it and its translation into three open reading frames. The first 300 bases encode the C-terminus of BLIP which ends at amino acid 88. The stop codon, TAA, is marked by an asterisk. The possible start codon for the *atp-A* ORF, M-112, is shown in bold. The *atp-A* and *atp-B* ORFs each contain one of the two Walker motifs. The conserved Walker A and B motifs as well as the S-G-R element that marks the putative loop 3 which brings Walker A and B together to form the ATP-binding cavity are underlined. It is unusual that Walker A and B appear on different polypeptides as this has not been seen in any other reported ATP-binding proteins. The amino acid sequence that is boxed and highlighted shows the regions most closely resembling the amino acid sequences of ATP-binding proteins in the literature. Figure 31 is an alignment of the predicted amino acid sequence of the

Figure 30. Sequence of the area downstream of the *bli* gene. The conserved amino acid elements making up the Walker A and Walker B motifs and the conserved loop 3 are highlighted and underlined. Proposed amino acid sequences are highlighted. Regions showing ambiguity due to possible frame shifts are boxed. The putative start codon for the *atp-A* gene, M-112, is shown in bold. The start codon for the *atp-B* ORF is unknown. A possible ATP-B amino acid sequence begins at the H-218. Putative promoter regions for the gene encoding the MSD is underlined. Asterisks indicate stop codons.

```
700
/1                                      731/11
CGA GCG CCC CGA CGC TCA CCC TCG CCA AGT TCA ACC AGG TCA CCG TGG GGA TGA CCA GGG
 S   A   P   T   L   T   L   A   K   F   N   Q   V   T   V   G   M   T   R   A

761/21                                  791/31
CCC AGG TAC TGG CGA CCG TCG GGC AGG GGT CCT GCA CCA CCT GGA GTG AGT ACT ACC CGG
 Q   V   L   A   T   V   G   Q   G   S   C   T   T   W   S   E   Y   Y   P   A

821/41                                  851/51
CCT ATC CGT CGA CGG CCG GGG TGA CCC TCA GCC TGT CCT GCT TCG ATG TGG ACG GTT ACT
 Y   P   S   T   A   G   V   T   L   S   L   S   C   F   D   V   D   G   Y   S

881/61                                  911/71
CGT CGA CGG GGT TCT ACC GAG GCT CGG CGC ACC TCT GGT TCA CGG ACG GGG TGC TTC AGG
 S   T   G   F   Y   R   G   S   A   H   L   W   F   T   D   G   V   L   Q   G

941/81                                  971/91
GCA AGC GGC AGT GGG ACC TTG TAT AAG GAC GCG GTT TCA CTG TGC GGG GCG GAT CAC CCG
 K   R   Q   W   D   L   V   *

1001/101                               1031/111
GTG ATC CGC CCC GCA CGG CCA TGA ACC AGG GGG ATG GGA AAC ATC AGT ACG TCA GAA ATT
V   I   R   P   A   R   P   *   T   R   G   M   G   N   I   S   T   S   E   I
 *   S   A   P   H   G   H   E   P   G   G   W   E   T   S   V   R   Q   K   F
  D   P   P   R   T   A   M   N   Q   G   D   G   K   H   Q   Y   V   R   N   S

1061/121                               1091/131
CTC GTC GCC GAA GGA GTC GAC CTC TCC TAC GGC GAT CAG CCC GCC GTG CGG GAT GCC CGG
L   V   A   E   G   V   D   L   S   Y   G   D   Q   P   A   V   R   D   A   R
 S   S   P   K   E   S   T   S   P   T   A   I   S   P   P   C   G   M   P   G
  R   R   R   R   S   R   P   L   L   R   R   S   A   R   R   A   G   C   P   D

1121/141                               1151/151
ATC TCG GTG GTA CCG GGG GAG GTG GTC GCG ATC ACC GGG CAG AGC GGG TCC GGG AAG TCG
I   S   V   V   P   G   E   V   V   A   I   T   G   Q   S   G   S   G   K   S
 S   R   W   Y   R   G   R   W   S   R   S   P   G   R   A   G   P   G   S   R
  L   G   G   T   G   G   G   G   R   D   H   R   A   E   R   V   R   E   V   V

1181/161                               1211/171
TCG CTC CTC TAC TGC CTG GCG GGG GTG GTG CCC GTC AGC CGG GGG CGG GTG CGG TTC GAG
S   L   L   Y   C   L   A   G   V   V   P   V   S   R   G   R   V   R   F   E
 R   S   S   T   A   W   R   G   W   C   P   S   A   G   G   G   C   G   S   R
  A   P   L   L   P   G   G   G   G   A   R   Q   P   G   A   G   A   V   R   G

1241/181                               1271/191
GGG CGC GTC ATC GGG GGG ATG TCC GAC GAC GAG GTC AGC GCG CTG CGG CGG GAA CGG TTC
G   R   V   I   G   G   M   S   D   D   E   V   S   A   L   R   R   E   R   F
 G   A   S   S   G   G   C   P   T   T   R   S   A   R   C   G   G   N   G   S
  A   R   H   R   G   D   V   R   R   R   G   Q   R   A   A   A   G   T   V   R

1301/201                               1331/211
GGA TTT GTC TTC CAG TAC GGG GAG TTG CTT CCC GAG CTG ACC ATC GAG GAG AAC ACC GCG
G   F   V   F   Q   Y   G   E   L   L   P   E   L   T   I   E   E   N   T   A
 D   L   S   S   S   T   G   S   C   F   P   S   *   P   S   R   R   T   P   R
  I   C   L   P   V   R   G   V   A   S   R   A   D   H   R   G   E   H   R   A

1361/221                               1391/231
CTG CCG CTG CGC CTG GCG GGC AGA GTC AGC GGG TCG CGG TGG CGC GGG CGC TGG TGC ACC
L   P   L   R   L   A   G   R   V   S   G   S   R   W   R   G   R   W   C   T
 C   R   C   A   W   R   A   E   S   A   G   R   G   G   A   G   A   G   A   P
  A   A   A   P   G   G   Q   S   Q   R   V   A   V   A   R   A   L   V   H   R
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1421/241                                1451/251
GGC CCG CCG TGG TCT TCG CCG ACG AGC CGA CCG GCT CAC TGG ACA GCG CCA ACG CCA CCG
G   P   P   W   S   S   P   T   S   R   P   A   H   W   T   A   P   T   P   P
 A   R   R   G   L   R   R   R   A   D   R   L   T   G   Q   R   Q   R   H   R
  P   A   V   V   F   A   D   E   P   T   G   S   L   D   S   A   N   A   T   A

1481/261                                1511/271
CCG TGC TGA AGG AGT TCC TCG GGC TGG CCC GTT CGC AGC GCA CGG CGG TGA TCC TGG TGA
P   C   *   R   S   S   S   G   W   P   V   R   S   A   R   R   *   S   W   *
 R   A   E   G   V   P   R   A   G   P   F   A   A   H   G   G   D   P   G   D
  V   L   K   E   F   L   G   L   A   R   S   Q   R   T   A   V   I   L   V   T

1541/281                                1571/291
CCC ACG ACG GCG CGG TGG CGG AGC AGG CGG ACA CCC GTT ACA CGA TGA CCG ACG GCG TCC
P   T   T   A   R   W   R   S   R   R   T   P   V   T   R   *   P   T   A   S
 P   R   R   R   G   G   G   A   G   G   H   P   L   H   D   D   R   R   R   P
  H   D   G   A   V   A   E   Q   A   D   T   R   Y   T   M   T   D   G   V   L

1601/301                                1631/311
TCT CCC CTG GGG GGA GCG GTG ACC GAG TTC CTG CTC GGA CTG CGG CTG CTC GCC GGT TCG
S   P   L   G   G   A   V   T   E   F   L   L   G   L   R   L   L   A   G   S
 L   P   W   G   E   R   *   P   S   S   C   S   D   C   G   C   S   P   V   R
  S   P   G   G   S   G   D   R   V   P   A   R   T   A   A   A   R   R   F   G

1661/321                                1691/331
GGG CGG GGC AAC CGC GTC CGC TTC CTG CTG ATG GCG GTC GGC GGG TCG GTC GGG GTC TGC
G   R   G   N   R   V   R   F   L   L   M   A   V   G   G   S   V   G   V   C
 G   G   A   T   A   S   A   S   C   *   W   R   S   A   G   R   S   G   S   A
  A   G   Q   P   R   P   L   P   A   D   G   G   R   R   V   G   R   G   L   L

1721/341                                1751/351
TGC CTG GCC CTC GTC CTC ACC CTT CCC GCG ATC CTC GAC GCG CAC AAC GGA CGG GCC GCG
C   L   A   L   V   L   T   L   P   A   I   L   D   A   H   N   G   R   A   A
 A   W   P   S   S   S   P   F   P   R   S   S   T   R   T   T   D   G   P   R
  P   G   P   R   P   H   P   S   R   D   P   R   R   A   Q   R   T   G   R   G

1781/361                                1811/371
GCC CGG CAG CCG GTG ACC GCG GCG AAG GGA CCG GCG GAC GGC ACC CTC GTG CTC CCG CGT
A   R   Q   P   V   T   A   A   K   G   P   A   D   G   T   L   V   L   P   R
 P   G   S   R   *   P   R   R   R   D   R   R   T   A   P   S   C   S   R   V
  P   A   A   G   D   R   G   E   G   T   G   G   R   H   P   R   A   P   A   F

1841/381                                1871/391
TCG GAC GCG TAC GGC TCC CAC TCG TTC ACC CGG GTC TTC GTG GCC CGA GGC CCG GGG CAG
S   D   A   Y   G   S   H   S   F   T   R   V   F   V   A   R   G   P   G   Q
 R   T   R   T   A   P   T   R   S   P   G   S   S   W   P   E   A   R   G   R
  G   R   V   R   L   P   L   V   H   P   G   L   R   G   P   R   P   G   A   G

1901/401                                1931/411
GAC ACC CCC GCC CCG CCC GGT CTG CGG GAA CTG CCC CGC CCC GGA GAG GTG TTC GTC TCC
D   T   P   A   P   P   G   L   R   E   L   P   R   P   G   E   V   F   V   S
 T   P   P   P   R   P   V   C   G   N   C   P   A   P   E   R   C   S   S   P
  H   P   R   P   A   R   S   A   G   T   A   P   P   R   R   G   V   R   L   P

1961/421                                1991/431
CCG CGC GTC CGT GAG CTG CTA CGG GAG GCG CCC GCC GTC AAG GGA CTG CTT CCC GGT GAT
P   R   V   R   E   L   L   R   E   A   P   A   V   K   G   L   L   P   G   D
 R   A   S   V   S   C   Y   G   R   R   P   P   S   R   D   C   F   P   V   M
  A   R   P   *   A   A   T   G   G   A   R   R   Q   G   T   A   S   R   *   *
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2021/441                                2051/451
GAG AAG GGG ACG ATC GGC CCC GGC GGT CTG GCC CAC CCC GAC GAG CTG TAC GCG TAC ATC
E   K   G   T   I   G   P   G   G   L   A   H   P   D   E   L   Y   A   Y   I
 R   R   G   R   S   A   P   A   V   W   P   T   P   T   S   C   T   R   T   S
  E   G   D   D   R   P   R   R   S   G   P   P   R   R   A   V   R   V   H   R

2081/461                                2111/471
GGC ACC ACC CGC GAC CGG CTG GAC GGC GAA GGA CGG CAC CTC AAG GGC TTC GGC TAC CGC
G   T   T   R   D   R   L   D   G   E   G   R   H   L   K   G   F   G   Y   R
 A   P   P   A   T   G   W   T   A   K   D   G   T   S   R   A   S   A   T   A
  H   H   P   R   P   A   G   R   R   R   T   A   P   Q   G   L   R   L   P   L

2141/481                                2171/491
TAC GCC CCG AAC CCG GTG GTC GAC CCG TCC ACC CTG ACG GAC GTA CGG TTC GCG CTG GCG
Y   A   P   N   P   V   V   D   P   S   T   L   T   D   V   R   F   A   L   A
 T   P   R   T   R   W   S   T   R   P   P   *   R   T   Y   G   S   R   W   R
  R   P   E   P   G   G   R   P   V   H   P   D   G   R   T   V   R   A   G   D

2201/501                                2231/511
ACC CTG GTC CTG CTG CCG CTG GGC ATC TTC CTC TCC GTC TGC GCC CGG CTG TCC GCG GCC
T   L   V   L   L   P   L   G   I   F   L   S   V   C   A   R   L   S   A   A
 P   W   S   C   C   R   W   A   S   S   S   P   S   A   P   G   C   P   R   P
  P   G   P   A   A   A   G   H   L   P   L   R   L   R   P   A   V   R   G   Q

2261/521                                2291/531
AGC CGC ACC CGC AGA CTG GCC TCG CTG CGG CTG CTG GGC CTG AGC AAG AAG GGC ACC CAG
S   R   T   R   R   L   A   S   L   R   L   L   G   L   S   K   K   G   T   Q
 A   A   P   A   D   W   P   R   C   G   C   W   A   *   A   R   R   A   P   S
  P   H   P   Q   T   G   L   A   A   A   A   G   P   E   Q   E   G   H   P   A

2321/541                                2351/551
CGG GTG AAC GCC GCC GAG ACC ACC GCC GCC GCG CTG ACC GGG GCG GCC CTC GGC CTG GGC
R   V   N   A   A   E   T   T   A   A   A   L   T   G   A   A   L   G   L   G
 G   *   T   P   P   R   P   P   P   P   R   *   P   G   R   P   S   A   W   A
  G   E   R   R   R   D   H   R   R   R   A   D   R   G   G   P   R   P   G   R

2381/561                                2411/571
GAG TAC ACG CTG CTC AAC CAG GTG ATG TCC CGG ACC GGG CTG CCC TCC CTG CGG TGG TAC
E   Y   T   L   L   N   Q   V   M   S   R   T   G   L   P   S   L   R   W   Y
 S   T   R   C   S   T   R   *   C   P   G   P   G   C   P   P   C   G   G   T
  V   H   A   A   Q   P   G   D   V   P   D   R   A   A   L   P   A   V   V   P

2441/581                                2471/591
CCC GAG GAC GGC GCG CTC TCC GCG ACC ACC GTC GCC GTC TGT CTG ATC GGC TGC CCG GCC
P   E   D   G   A   L   S   A   T   T   V   A   V   C   L   I   G   C   P   A
 P   R   T   A   R   S   P   R   P   P   S   P   S   V   *   S   A   A   R   P
  R   G   R   R   A   L   R   D   H   R   R   R   L   S   D   R   L   P   G   P

2501/601                                2531/611
CTC GCC TGG CTC GTC GGC CGC CGC AGC GCG CGG GAC GCC ACC GCC GAC CCG CTG GCC GTC
L   A   W   L   V   G   R   R   S   A   R   D   A   T   A   D   P   L   A   V
 S   P   G   S   S   A   A   A   A   R   G   T   P   P   P   T   R   W   P   S
  R   L   A   R   R   P   P   Q   R   A   G   R   H   R   R   P   A   G   R   P

2561/621                                2591/631
CGG CGC ACC GCC GTC GCG CGC CCG CCG ACC AAG TGG TTC GGC CTG CTG CTG CTC GCG GGC
R   R   T   A   V   A   R   P   P   T   K   W   F   G   L   L   L   L   A   G
 G   A   P   P   S   R   A   R   R   P   S   G   S   A   C   C   C   S   R   A
  A   H   R   R   R   A   P   A   D   Q   V   V   R   P   A   A   A   R   G   P
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2621/641                                2651/651
CTG GGA ATC GTC TGC GGC TAC TGC CTC ACG GGC CTG CTG GGA CGT CCC GCG TCC AGC GTC
L   G   I   V   C   G   Y   C   L   T   G   L   L   G   R   P   A   S   S   V
 W   E   S   S   A   A   T   A   S   R   A   C   W   D   V   P   R   P   A   S
  G   N   R   L   R   L   L   P   H   G   P   A   G   T   S   R   V   Q   R   R
2681/661                                2711/671
GGG GTG AAC GCG CTC CTC GTG GTC GCG GGT GTG CTG CTC ACC GGG GTC GGG CTG GTG CTC
G   V   N   A   L   L   V   V   A   G   V   L   L   T   G   V   G   L   V   L
 G   *   T   R   S   S   W   S   R   V   C   C   S   P   G   S   G   W   C   S
  G   E   R   A   P   R   G   R   G   C   A   A   H   R   G   R   A   G   A   H

2741/681                                2771/691
ACC CTG CCC TAT CTG TCG TAC GCG CTC GCC CGC GCG CTC GCG GGG TCG ACC CGT TCC CTC
T   L   P   Y   L   S   Y   A   L   A   R   A   L   A   G   S   T   R   S   L
 P   C   P   I   C   R   T   R   S   P   A   R   S   R   G   R   P   V   P   S
  P   A   L   S   V   V   R   A   R   P   R   A   R   G   V   D   P   F   P   H

2801/701                                2831/711
ACC CTC AAT CTG GCC ATG CGG CGC AAC GAG GCC GAG CCC GGC AGC ACC CTG CGG GTG GTC
T   L   N   L   A   M   R   R   N   E   A   E   P   G   S   T   L   R   V   V
 P   S   I   W   P   C   G   A   T   R   P   S   P   A   A   P   C   G   W   S
  P   Q   S   G   H   A   A   Q   R   G   R   A   R   Q   H   P   A   G   G   H

2861/721                                2891/731
ACG GGG CTG GTC CTG CTG GTG TAC GCC GCG TCC CTC GCT CAG GGG GTG CTC ATC CAG CTC
T   G   L   V   L   L   V   Y   A   A   S   L   A   Q   G   V   L   I   Q   L
 R   G   W   S   C   W   C   T   P   R   P   S   L   R   G   C   S   S   S   S
  G   A   G   P   A   G   V   R   R   V   P   R   S   G   G   A   H   P   A   R

2921/741                                2951/751
GAC CAG GTC AGC CGG CCC TCC GGC CCC GTT CAG GAC TAC TCC CTG GCC CTG CAC GAG CTG
D   Q   V   S   R   P   S   G   P   V   Q   D   Y   S   L   A   L   H   E   L
 T   R   S   A   G   P   P   A   P   F   R   T   T   P   W   P   C   T   S   *
  P   G   Q   P   A   L   R   P   R   S   G   L   L   P   G   P   A   R   A   D

2981/761                                3011/771
ACC GAG CGG CAG CGG CAG GAG CTG GGC GGA CTG GAC GGG GTC CGC ACC CGG GCC GTG ATG
T   E   R   Q   R   Q   E   L   G   G   L   D   G   V   R   T   R   A   V   M
 P   S   G   S   G   R   S   W   A   D   W   T   G   S   A   P   G   P   *   *
  R   A   A   A   A   G   A   G   R   T   G   R   G   P   H   P   G   R   D   D

3041/781                                3071/791
ATG AAC TCC TGG GTG GAT CTG CGG GCG GAG AGC CCC GAC GCG GCG TTC GCC TCG TCG GCG
M   N   S   W   V   D   L   R   A   E   S   P   D   A   A   F   A   S   S   A
 *   T   P   G   W   I   C   G   R   R   A   P   T   R   R   S   P   R   R   R
  E   L   L   G   G   S   A   G   G   E   P   R   R   G   V   R   L   V   G   D

3101/801
ACC GCC CTG GTC GCC ACC TGC GAT GAG
T   A   L   V   A   T   C   D   E
 P   P   W   S   P   P   A   M
  R   P   G   R   H   L   R   *
```

Figure 31. Alignment of the amino acid sequences of ATP-A and ATP-B of *S. clavuligerus* with an ATP-binding protein sequence of a *Streptomyces coelicolor* ABC transporter. Plus (+) indicate similar residues. Numbers at the beginning and end of each line indicate the position of the amino acid residue within the protein. Dashes between amino acid residues represent gaps introduced to maximize sequence alignment.

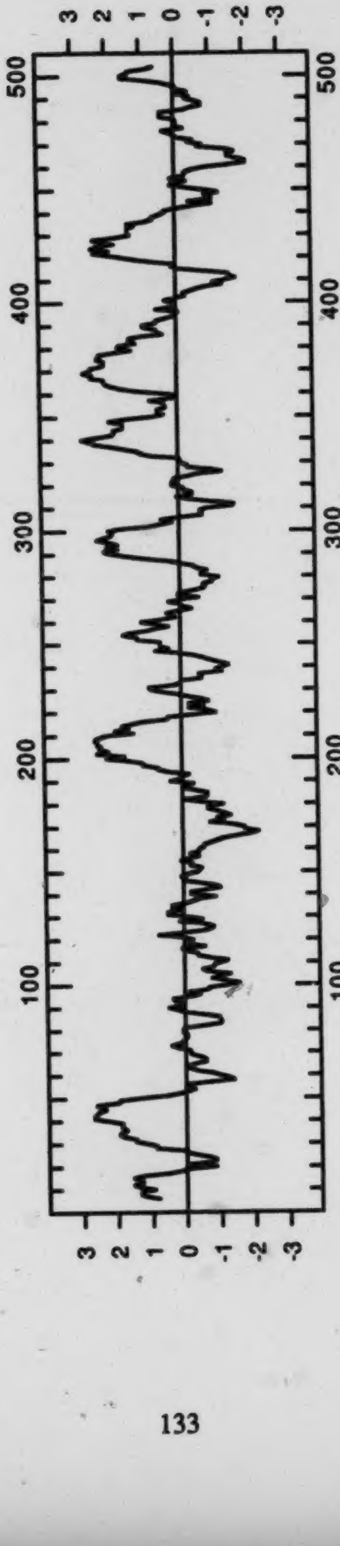
```
ATP-A          115 ISTSEILVA-EGVDLSYGDQP----AVRDARISVVPEEVVATPGQSGSGKSSLLNCLAGV 169
                   +S++++LV+  GV L++G  P    AV  A + V   + +A  G SGSGKSSLL+ LAG+
S.coelicolor     1 MSSADLLVSCRGVALTFGRGPTAVVAVHGADLDVRCGDRLAVVGPSGSGKSSLLHLLAGL 60

ATP-A          170 VPVSRGRVRFEGRVIGGMSDDEVSALRRERFGFVFQYGELLPELTIEENTALPLRLA 226
                      + G +   G  + G  D           G VFQ   L+P L + ENTALPL LA
S. coelicolor   61 ERPTSGTITRPG--LHGPRD----------IGLVFQADSLIPALDVTENTALPLVLA 105


ATP-B          225 GGQSQRVAVARALVHRPAVVFADEPTGSLDSANATAVLKEFLGLARSQRTAVILVTHDGAVAEQADTRYTMTDGVL 1602
                   GGQ+QRVAVAR L   P ++ ADEPTG LD A    VL   L  A     A+++ THD AVA +   R  M DG L
S. coelicolor  138 GGQAQRVAVARVLAQAPRLILADEPTGRLDHATGARVLDALLAAADRTGAALVVTTHDPAVAARLTLRRGMRDGRL 300
```

atp-A ORF and the *atp-B* ORF with the ATP-binding domain of a known *Streptomyces coelicolor* ABC transporter protein (Redenbach *et al.*, 1996). Although the Walker elements are found in all such proteins, the sequence identity of the ATP-binding domains is not limited to just these elements. Rather, high sequence identity occurs throughout the entire ATP-binding domain of these proteins (Higgins, 1992). There is 45 % identity and 57% similarity between the *S. clavuligerus* and the *S. coelicolor* protein.

The location of the two Walker motifs on separate reading frames is unusual and suggests that a frame shift occurred in the region between these conserved motifs. Since no sequencing error was found upon repeated sequencing of both strands, it was concluded that a deletion had taken place in this area. The second evidence of a deletion event in this region is the observation that the distance between the lysine residue (K) in Walker A and the aspartate residue (D) in Walker B is 88 amino acids apart while in all other reported ABC transporters in *Streptomyces*, the distance between these two absolutely conserved residues ranged between 116-149 amino acids apart (Mendez and Salas, 1998). The deletion event could then have resulted in the removal of a segment of amino acids joining Walker A and Walker B motifs. The resulting frame shift would then appear as two ORFs. No stop codon was identified between the *atp-A* and *atp-B* ORFs nor following the *atp-B* ORF. A PCR reaction designed to amplify the junction between the observed Walker A and B motifs using genomic DNA as a template would determine if a deletion event had taken place in this area. It is also possible that this gene has no function in *S. clavuligerus* and is not expressed. Analysis of the transcript would also show if this is a functional gene as well as resolve the question of the possible deletion event.

A large ORF was identified in the region following the *atp-A* and *atp-B* genes. Although a possible promoter region indicated by the underlining and a putative start codon, V-307, is proposed, since no stop codon was identified for the preceeding *atp-A/B*, it is possible that the ATP-binding domain is fused to this second ORF, believed to encode the MSD. Nucleotides 1601 to the end of the sequence (Figure 30) were translated and the amino acid sequence was subjected to hydrophobicity analysis. The Kyte Doolittle hydrophobicity plot shown in Figure 32 revealed the presence of possible multiple membrane spanning domains. Like the MSDs of other ABC transporters, this incomplete gene showed homology to no known protein in the data base. Two possible

Figure 32. Kyte Doolittle hydrophobicity plot of the amino acid sequence of the MSD. The incomplete ORF downstream of the *atp-A* and *atp-B* genes was analysed for the presence of hydrophobic amino acid domains. Hydrophobic domains are shown above the midpoint line while domains containing amino acids that have hydrophilic side chains are shown below the midpoint line.


100
200
300
400
500
3
2
1
0
-1
-2
-3

frame shifts may have taken place in the first 200 base pair of the gene. This is shown by the clear box and is as yet unresolved.

VI. RESULTS: NUTRITIONAL STUDIES.

Little is known about the regulation of BLIP production by *S. clavuligerus*. It is known that BLIP is produced in trypticase soy/starch medium (TSBS), a complex medium containing a digest of casein and soybean, and in GSPG medium, a defined medium containing glycerol, sucrose, L-proline and L-glutamate. BLIP cannot be detected in starch asparagine (SA) medium, a defined medium containing starch as the carbon source and L-asparagine as the nitrogen source. In *S. exfoliatus*, the other *Streptomyces* species which produces two exocellular proteins having β-lactamase inhibitory activity, BLIP-I and BLIP-II are produced in a defined medium containing glucose as a carbon source and NH_4Cl as a nitrogen source (Kim *et al.*, 1994). One objective of the nutritional studies experiments described in this chapter was to determine if BLIP production in *S. clavuligerus* can be induced by modifying the carbon or nitrogen sources of SA medium and to compare SA and GSPG media to determine which component(s) of GSPG are critical for BLIP production.

Secondly, from the *bli* gene disruption work (Results Section III), a transient increase in clavam production was seen in the *bli* and *bli/claR* mutants. Genetic mapping experiments (Results Section IV) suggest that the *bli* gene may be linked to genes involved in clavam biosynthesis. For these reasons, a comparison of BLIP production with clavam production was undertaken to determine if there were similarities in their expression in different media.

The third question that was addressed in these experiments was whether the production of BLIP was affected in a *ccaR* mutant. *ccaR* encodes a transcriptional activator shown to be essential for production of β-lactam products (Perez-Llarena and Liras *et al.*, 1997). The level of production of BLIP would be expected to be altered in a *ccaR* mutant if the *bli* gene is regulated in a similar manner to the β-lactam products of *S. clavuligerus*.

VI.1 EFFECT OF GROWTH MEDIUM COMPOSITION ON PRODUCTION OF A HEAT LABILE β-LACTAMASE INHIBITOR.

VI.1.1 Effect of Modifications of Carbon and Nitrogen Sources in SA Medium on BLIP Production.

Preliminary studies in which GSPG culture supernatants were assayed for inhibitory activity against Bactopenase showed that a heat labile β-lactamase inhibitor, presumably BLIP, could be detected. Since this activity appeared to be produced in GSPG and not in SA medium and yet both are defined media, derivatives of SA medium containing various combinations of carbon and nitrogen sources were used to determine if production was an effect of a particular carbon source or a specific amino acid. Tables V and VI describe the various derivatives of SA media used in BLIP production studies.

In Table V, starch and L-asparagine were replaced with glycerol and NH_4Cl. NH_4Cl was chosen in place of L-asparagine because it was the nitrogen source in the *S. exfoliatus* BLIP-I and BLIP-II production medium (Kim *et al.*, 1994). Glycerol was chosen as an alternative carbon source for two reasons: it was readily available and *S. clavuligerus* does not grow well in sucrose containing medium; *S. clavuligerus* also does not metabolized glucose (Garcia-Dominguez *et al.*, 1989). Duplicate 100 ml amounts of each medium were inoculated with a spore suspension of wild type *S. clavuligerus*. The cultures were incubated at 28 °C with shaking at 280 rpm. One set of cultures was harvested at 4 days and the second set was harvested at 7 days post inoculation. The optical densities of the cultures were determined at the time of harvest. Bactopenase inhibition assays were carried out on the culture supernatants to determine if BLIP was produced.

Each culture supernatant sample was assayed with and without heat inactivation. β-lactamase inhibitory activity values obtained for samples that had not been heat inactivated represent the sum activity of BLIP and clavulanic acid present. β-lactamase inhibitory activity values obtained for the heat inactivated samples were taken to be a measure of clavulanic acid alone. The difference (Δ% inhibition) between the values obtained for a sample that was heat inactivated and the same sample without heat inactivation represented BLIP activity. Since clavulanic acid was produced in abundance in SA cultures, culture supernatant samples from SA cultures were used as the negative controls. It was presumed that clavulanic acid was sufficiently heat stable as the heat inactivation step seemed to have no effect on β-lactamase inhibitory activity of SA culture supernatants and so any change in inhibition was a measure of BLIP activity. A percent inhibition below 15% was considered negative since duplicate assays on the same sample that was not expected to contain BLIP could vary as much as 12%.

Table V. Effect of carbon and nitrogen on the production of β-lactamase inhibitors in *S. clavuligerus* cultures grown in SA-based media.

	carbon source		**nitrogen source**				**% inhibition due to[b]:**	
	starch (1 % w/v)	glycerol (1.5 % w/v)	L-asparagine (0.2 % w/v)	NH_4Cl (0.2 % w/v)	age of culture	OD_{600}	clavulanic acid	BLIP
Medium[a]								
1		x	x		4 days	0.85	16.6	< 15
2		x		x	4 days	0.72	0	< 15
3	x		x		4 days	4.0	51.6	< 15
4	x			x	4 days	0.7	0	< 15
1		x	x		7 days	2	48	< 15
2		x		x	7 days	2	57	< 15
3	x		x		7 days	6	60	< 15
4	x			x	7 days	1.2	34	< 15

[a] Media 1 through 4 are SA-based media in which starch and/or L-asparagine have been replaced by glycerol or NH_4Cl. Medium 3 is the original SA medium. Duplicates of each medium were inoculated with a spore suspension of wild type *S. clavuligerus* and one set was grown for 4 days while the second set was grown for 7 days at which time the cultures were harvested and the OD_{600} determined.

[b] Percent inhibition against the activity of Bactopenase in the degradation of Penicillin G was determined for each of the samples. Inhibition due to clavulanic acid alone was determined by assaying samples that have been subjected to heat inactivation. Inhibition due to BLIP was determined by taking the difference between the untreated and the heat inactivated samples. An inhibition value greater than 15 % is required to be considered positive.

Table VI. Effective of carbon and nitrogen sources on production of BLIP by *S. clavuligerus* cultures grown in SA-based media.

	carbon source			nitrogen source			
Medium[a]	starch (1 % w/v)	glycerol (1.5 % w/v)	sucrose (2 % w/v)	proline (0.25 % w/v)	glutamic acid (0.15 % w/v)	**OD_{600}**[b]	**Δ % inhibition**[c]
1		x	x	x	x	0.49	< 15 %
2	x		x	x	x	3.4	< 15 %
3		x		x	x	0.68	< 15 %
4	x			x	x	2.9	< 15 %
5		x	x		x	0.8	< 15 %
6	x		x		x	1.8	< 15 %
7		x	x	x		0.04	< 15 %
8	x		x	x		0.24	< 15 %
9		x			x	0.45	< 15 %
10	x				x	1.4	< 15 %
11		x		x		0.03	< 15 %
12	x			x		0.22	< 15 %

[a] Media 1-12 are SA based media in which starch and L-asparagine have been replaced by the carbon and nitrogen sources of GSPG.

[b] OD_{600} of 72 hour cultures were determined at time of harvest.

[c] Supernatants of 72 hour cultures were assayed for BLIP activity by determining the difference (Δ) between rates of Bactopenase mediated Penicillin G degradation in the presence of samples that had been heat inactivated and rates obtained with samples that had not been heat treated. Results of Bactopenase inhibition assays are expressed as a % of the uninhibited reaction and are given as % inhibition. Percent inhibition was calculated as the average of 2 assays done on the same sample.

In Table V, the OD_{600} results show that the cultures grew poorly in all of these media with the exception of the original SA medium (sample # 3). In four day cultures, clavulanic acid production was not seen in media 2 and 4. Medium 1 and the original SA medium, medium 3, supported clavulanic acid production by day 4. All cultures produced clavulanic acid by 7 days. BLIP activity was never seen in any of these cultures at either time point showing that neither glycerol nor NH_4Cl had an effect on the detection of BLIP production in SA-based media.

In Table VI, starch and L-asparagine of SA medium were substituted with glycerol, sucrose, L-proline and L-glutamate from GSPG medium. The resulting twelve SA-based media described in Table VI were inoculated with spore suspensions of wild type *S. clavuligerus* and grown as described above for 72 hours. At this time the culture supernatants were collected and assayed for β-lactamase inhibitory activity with and without heat inactivation. The Δ% inhibitions in Table 6 show that replacing starch and L-asparagine of SA medium with the carbon and nitrogen sources of GSPG did not stimulate BLIP production in *S. clavuligerus* cultures grown in SA-based media. The corresponding optical densities showed that with the exception of cultures 2 and 4, most cultures did not grow well as was the case with most four day old cultures described in Table V. Correspondingly, no significant amounts of clavulanic acid could be detected by enzyme assays in these 72 hour cultures (data not shown). Cultures 2 and 4 reached optical density values of 3 which were comparable to wild type SA cultures under similar conditions of growth. In these two cultures, the replacement of L-asparagine by L-glutamate or L-proline did not stimulate BLIP production in SA medium.

From the experiments described in Tables V and VI, it appeared that BLIP activity could not be detected in SA grown cultures regardless of the carbon and nitrogen sources of the medium.

VI.1.2 Effect of Modifications of the Carbon and Nitrogen Sources of GSPG Medium on BLIP Production.

The carbon and nitrogen sources of GSPG were deleted from GSPG and replaced with alternatives in order to determine if there was one component that was essential for BLIP production. Like the experiments described in the previous section, 100 ml amounts of each of the medium described in Table VII were inoculated with a spore suspension of wild type *S. clavuligerus* and grown for 72 hours at which time the

Table VII. Effect of carbon and nitrogen sources on production of a β-lactamase inhibitor in GSPG-based media

	carbon source			**nitrogen source**			
	starch (1 % w/v)	glycerol (1.5 % w/v)	sucrose (2 % w/v)	proline (0.25 % w/v)	glutamic acid (0.15 % w/v)		
Medium[a]						**OD_{600}**[b]	**Δ % inhibition**[c]
1		x	x	x	x	5.6	63
2		x	x	x		1.1	3.4
3	x		x	x	x	4.2	51
4	x		x	x		1.3	1.5
5		x		x	x	4.6	51
6	x			x	x	3.8	58
7		x	x		x	3.1	46
8	x		x		x	3.5	35

[a] Medium 1 is GSPG medium. Media 2-8 are GSPG media containing the indicated C and N sources.

[b] OD_{600} of 72 hour cultures at time of harvest.

[c] Supernatants of 72 hour cultures were assayed for BLIP activity by determining the difference between the rate of Bactopenase mediated Penicillin G degradation in the presence of samples that had been heat inactivated and rates obtained with samples that had not been heat treated. Initially believed to represent BLIP activity, the Δ % inhibition was found to be a sum of BLIP and clavulanic acid when methanol dilution was used instead of heat inactivation to remove the effect of BLIP activity. Percent inhibition was calculated from the average of 2 assays done on the same sample.

cultures were harvested by centrifugation and the supernatants assayed for BLIP activity. The Δ% inhibitions show that in GSPG media 2 and 4 which did not contain glutamic acid, no effect on the rate of Penicillin G degradation was observed when the supernatants of these cultures were assayed for Bactopenase inhibitory activity with or without heat inactivation. In the remaining media, differences in % inhibitions between the heat inactivated and the untreated samples were observed. Initially, the Δ % inhibitions in samples 1, 3, and 5-8 were presumed to be BLIP activity which then suggested that in GSPG medium, glutamic acid appeared to be the critical component in induction of BLIP production. However, the results of β-lactamase inhibitory activity assays with the heat inactivated samples showed no inhibition suggesting that no clavulanic acid activity was present in these culture supernatants. Since clavulanic acid is known to be produced in GSPG medium as determined by HPLC analysis by A. Wong, A. Paradkar and S. E. Jensen, (personal communication), the inability to detect it by enzyme assays in these samples suggested that clavulanic acid was being inactivated during the heat inactivation step. Prior to this experiment, clavulanic acid was presumed to be stable to the heat treatment because when SA culture supernatant was subjected to heat inactivation, no change in % inhibition was ever detected. It was unlikely that the inability to detect clavulanic acid was due to the cultures not having grown sufficiently to produce clavulanic acid since the optical density values of samples 1, 3, and 5 through 8 were 3.1 to 5.6, comparable to the value of 4.0 attained by a 4-day old SA culture that produced clavulanic acid (Table VI).

If clavulanic acid of GSPG was being inactivated by the heat treatment, then the apparent stability of clavulanic acid to heat inactivation in SA culture supernatants, the negative control, would be explained in two ways. First, the amount of clavulanic acid inactivated by the heat treatment in SA samples could be an insignificant amount of the total present in the supernatant and would not be detected perhaps due to the sensitivity limit of the assay. In GSPG samples on the other hand, perhaps the amount inactivated by heat treatment was a more significant percentage of the total. It was also possible that the 2.1% MOPS in the SA medium that was not present in GSPG increased the heat stability of clavulanic acid in the sample. The finding that clavulanic acid interfered with BLIP activity determination made interpretations of BLIP activity assay results of GSPG samples difficult; however the presence of BLIP in GSPG culture supernatants would subsequently be shown by western analysis. For this reason, it was likely that the % inhibition values presumed to be due to BLIP in GSPG culture supernatant samples described in Table VII more correctly reflected a sum activity of clavulanic acid and BLIP.

This finding that heat inactivation in certain cases (GSPG culture supernatants) could not be used to remove BLIP activity without also removing clavulanic acid activity made interpretations of BLIP assays difficult. For this reason, methanol inactivation was used in place of heat inactivation for determination of BLIP activity by Bactopenase inhibition assay. In methanol inactivation, a sample to be assayed was diluted with methanol and then the rate of Penicillin G degradation by Bactopenase was measured and compared with the rate obtained for a similar sample diluted with water. The contribution of BLIP is removed by dilution with methanol and the rate so obtained represented clavulanic acid activity only (Doran *et al.*, 1990). As in the heat inactivation method, the difference (Δ% inhibition) between the rates obtained for samples diluted with methanol and those diluted with water represented BLIP alone. In addition to Bactopenase inhibition assays, western immunodetection was also developed for the analysis of BLIP production under various nutritional conditions since western analysis gave more definite results as BLIP assays were often affected by components in the media.

In the next experiment, western analysis in addition to Bactopenase inhibition assays were used to determine if BLIP was produced in various combinations of SA and GSPG media that differed in buffer compositions, trace elemental salts as well as carbon and nitrogen sources.

VI.2 EFFECT OF BUFFER AND TRACE ELEMENTS ON PRODUCTION OF BLIP IN GSPG AND SA COMBINATION MEDIA.

In addition to differences in carbon and nitrogen sources, SA and GSPG have slightly different compositions of trace elemental salts (GSPG trace elements contain $FeCl_3 \cdot 6H_2O$ and $ZnCl_2$ instead of $FeSO_4 \cdot 7H_2O$ and $ZnSO_4 \cdot 7H_2O$ found in SA trace salts). Furthermore, the concentrations of all trace elements are approximately 100 fold higher in GSPG than in SA medium. A third difference is the presence of 2.1% MOPS buffer present in SA and absent from GSPG. The composition of GSPG and SA can then be divided into three components: (1) the carbon and nitrogen sources, (2) the type and amounts of trace elements and (3) buffer compositions. Twelve media, composed of various combinations of the carbon/nitrogen sources, trace elements and buffer components of GSPG and SA were made and tested for their ability to support BLIP production with the objective of identifying the component(s) in GSPG that were necessary for BLIP production or identifying the component(s) of SA medium that were inhibiting production. Since BLIP is produced in the later stages of growth, the cultures were harvested when a thick mycelial suspension was obtained. Because the different media supported growth to various degrees, the cultures were harvested at different times and optical densities were determined at harvest. Table VIII shows the compositions of the twelve media, the age of the culture at harvest and growth as indicated by optical density. Table IX summarizes the contents of BLIP and clavulanic acid produced by the respective cultures. The presence or absence of BLIP, as determined by standard Bactopenase inhibitory activity assays with and without methanol dilution, is expressed as + or - and these results are found in the "undialysed" column. (A % inhibition greater than 20% was considered positive for BLIP activity, lower than 15% was considered negative while marginal activity was a value in between.) Although the detection of BLIP activity was consistent and reproducible in medium 1, the original GSPG medium, % inhibitions varied greatly among replicate experiments. Marginal β-lactamase inhibitory activity was detected in media 3, 5 and 7 although this activity was not consistently seen in all three replicates of this experiment. Western analysis for BLIP in all the culture supernatants was carried out to determined if BLIP was present especially in samples where the presence of BLIP activity was not reproducible and to confirm that the inhibitory activity seen in sample 1 was due to BLIP.

Western analysis of the same culture supernatants was done as described in Materials and Methods. The results in Figure 33 A show that the mature BLIP was present in most combinations of GSPG/SA media. In lane 1 which corresponds to proteins from GSPG culture supernatant, a single band corresponding to the BLIP protein was seen confirming β-lactamase inhibition assay results. Surprisingly, proteins from SA culture supernatant (lane 10), also showed a mature BLIP protein, but a proteinaceous β-lactamase inhibitor has never been detected in SA culture supernatants by β-lactamase inhibition assays. It is believed that BLIP is expressed in SA cultures since the *bli* promoter is known to be active in SA-cultures (A. Paradkar, personal communication). In media 2, 4, 6, 8, and 12 which are low salt media, i.e. containing SA trace elements, the BLIP band tends to be fainter with an additional signal migrating between the 18.7 and 28 kDa size markers. It is not clear what this higher Mr signal is,

Table VIII. Compositions of media derived from SA and GSPG media used in BLIP production experiment.

Medium[a]	Buffer		trace elements		C/N source			Growth[b]	
	GSPG	SA	GSPG	SA	GSPG	SA	SG	OD_{600}	age of culture (hrs)
1:GSPG/GSPG/GSPG	x		x		x			11	52
2:GSPG/SA/GSPG	x			x	x			4.7	96
3:GSPG/GSPG/SA	x		x			x		13.1	52
4:GSPG/SA/SA	x			x		x		6.5	96
5:GSPG/GSPG/SG	x		x				x	4.7	140
6:GSPG/SA/SG	x			x			x	3.5	140
7:SA/GSPG/GSPG		x	x		x			12.5	52
8:SA/SA/GSPG		x		x	x			4.9	96
9:SA/GSPG/SA		x	x			x		9.7	52
10:SA/SA/SA		x		x		x		6.5	96
11:SA/GSPG/SG		x	x				x	5	140
12:SA/SA/SG		x		x			x	3.7	140

[a] The compositions of GSPG and SA media were divided into three components: (A) the buffer and Mg^{2+}, (B) trace elements and (C) the C and N sources. These components from GSPG and SA were mixed in different combinations to create 12 media used in this study. (Media designations are in the format A/B/C indicating whether the components are from the original SA or GSPG medium. SG medium contains starch and glutamate as the carbon and nitrogen sources respectively.) Each medium was inoculated with a 4% (v/v) TSBS seed inoculum as described in Materials and Methods. The cultures were incubated with shaking at 280 rpm at 28 °C until maximum growth was obtained.

[b] Age of cultures and corresponding OD_{600} attained at time of harvest.

Table IX. Effects of buffers, trace elements and C/N sources on production of BLIP by *S. clavuligerus*.

		BLIP		
Medium[a]		Bactopenase inhibition (% inhibition)		western analysis[d]
	µg protein	undialyzed[b]	dialyzed & concentrated[c]	
1:GSPG/GSPG/GSPG	12	+	69 %	++++
2:GSPG/SA/GSPG	19	-	-	+
3:GSPG/GSPG/SA	12	(+)	31 %	+
4:GSPG/SA/SA	19	-	-	-
5:GSPG/GSPG/SG	8	(+)	55 %	+++
6:GSPG/SA/SG	14	-	-	-
7:SA/GSPG/GSPG	11	(+)	56 %	++++
8:SA/SA/GSPG	12	-	-	+
9:SA/GSPG/SA	16	-	-	+++
10:SA/SA/SA	17	-	-	++
11:SA/GSPG/SG	10	-	-	++
12:SA/SA/SG	13	-	-	+

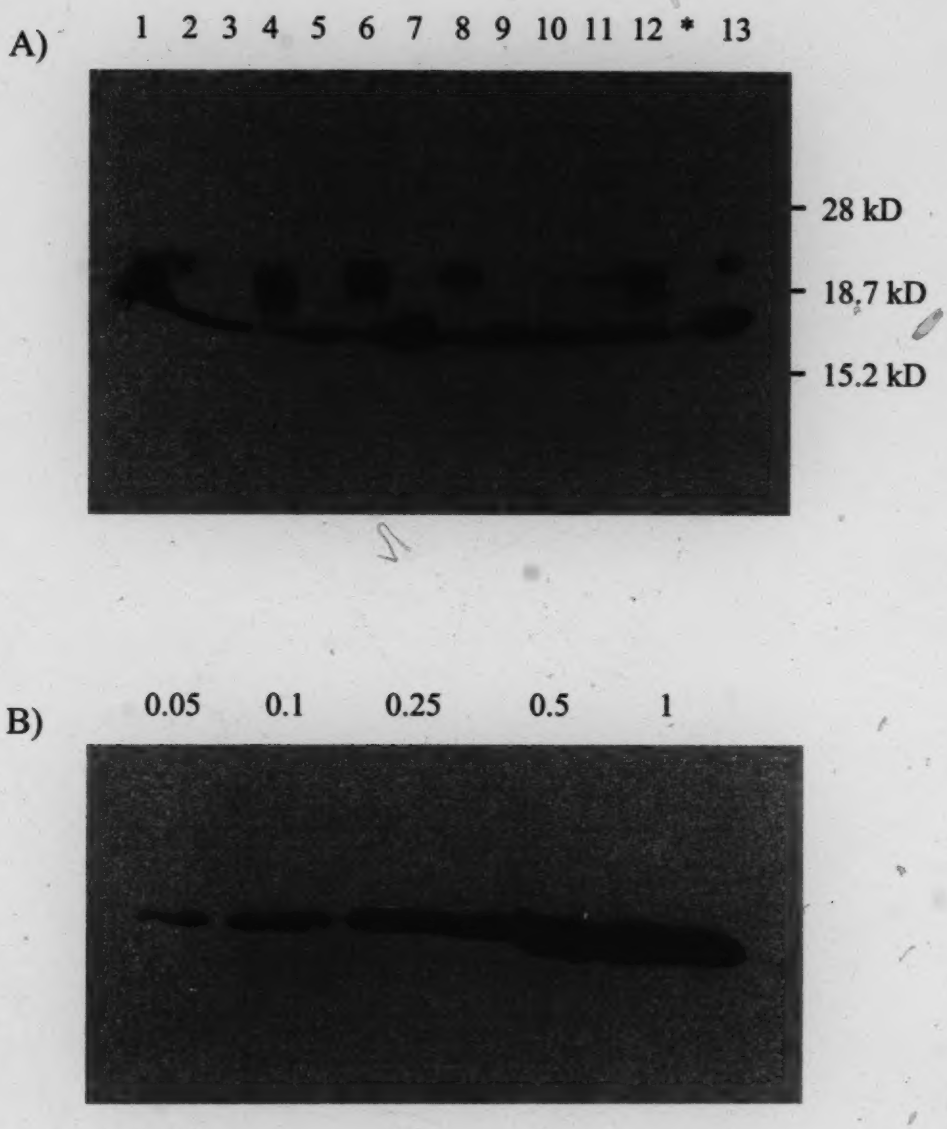
[a] Media 1 and 10 are the original GSPG and SA media. Compositions of the remaining media, inoculum and culture conditions are also described in Table VIII.

[b] Δ % inhibitions obtained when 100 µl of culture supernatants containing the respective proteins in µg amounts, were assayed for Bactopenase inhibitory activity with and without prior dilution with methanol. A + reaction indicates that a rate > 15 % was obtained. (+) indicates that inhibitory activity was seen although not in all three replicates of the experiment.

[c] % inhibition obtained when 500 µl of culture supernatant was dialyzed three times against 10 mM PO_4 pH 7 and concentrated to 1/5 the original volume.

[d] Proteins from culture supernatants were TCA precipitated, separated on a SDS-PAGE and analysed by western immunodetection as described in Materials and Methods. Each lane contained 40 µg of proteins. (See Figure 33).

Figure 33. A) Western analysis of supernatants from cultures grown in GSPG/SA combination media (described in Table IX) for the presence of the BLIP protein. Samples from GSPG/SA media 1-12 were precipitated with 10% TCA and 40 μg of proteins per lane were separated on a 12% SDS-PAGE. The proteins were then blotted onto a PVDF membrane and the presence of BLIP proteins was detected by hybridization with BLIP antibodies. Lanes 1-12 represented proteins from media 1 through 12 respectively. The lane labelled with the asterisk contained standard protein size markers while lane 13 contained proteins from wild type *S. clavuligerus* TSBS culture supernatants and served as the positive control. B). Western analysis of purified BLIP for determining the detection limit of western immunodetection. Various amounts from 0.05 to 1 μg of purified BLIP were run on a 12% SDS-PAGE, the proteins were blotted onto a PVDF membrane and western analysis using primary BLIP antibodies was done.


A)
1 2 3 4 5 6 7 8 9 10 11 12 * 13
28 kD
18.7 kD
15.2 kD
B)
0.05 0.1 0.25 0.5 1

but since the antibodies used in the western analysis is specific for the BLIP protein, (a later figure will show that this signal was not seen in the culture supernatants of the *bli* mutant), it is possible that this signal corresponds to unprocessed forms of the BLIP protein. Culture supernatant samples in which the medium consisted of higher salt GSPG trace elements (samples 1, 3, 5, 7, and 9) did not show this additional signal; instead, a more intense BLIP band was seen. The relative intensities of the BLIP band for the different samples are also included in Table IX.

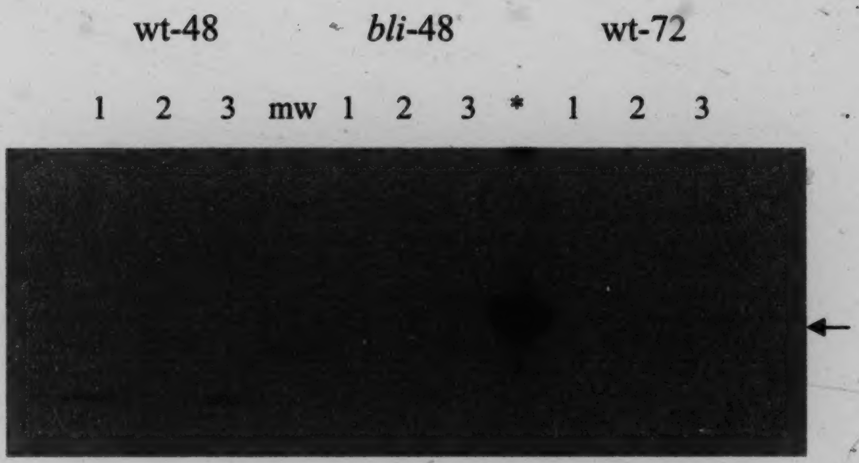
Comparison of the western hybridization results and the enzyme inhibition assay results shown in the "undialyzed" column show that the presence of a signal by western analysis did not always correlate to detectable activity. Although β-lactamase inhibition assays did not always give consistent results (samples 3, 5 and 7) the results of western analysis were consistent and reproducible with respect to the presence of the BLIP signal in most samples including SA and the presence of a stronger signal in the low salt media (even number samples). Western analysis and Bactopenase inhibition assays of purified BLIP were done to compare the detection limits of each method. Bactopenase assays were done with decreasing amounts of purified BLIP starting at 1 mg of protein until Bactopenase inhibitory activity could no longer be detected. The limit of the Bactopenase inhibition assay was determined to be between 0.1 µg and 0.05 µg of protein as BLIP activity could be detected at 0.1 µg but not at 0.05 µg. Similar amounts of purified proteins were analysed on a western blot hybridized against BLIP antibodies. Figure 33 B shows that BLIP can be readily detected at 0.05 µg of protein suggesting that western analysis was a more sensitive technique than Bactopenase inhibition assay for the detection of BLIP. It was possible then that the discrepancy of western analysis with Bactopenase inhibition assay results could be explained by a difference in the detection limit of the two assay methods.

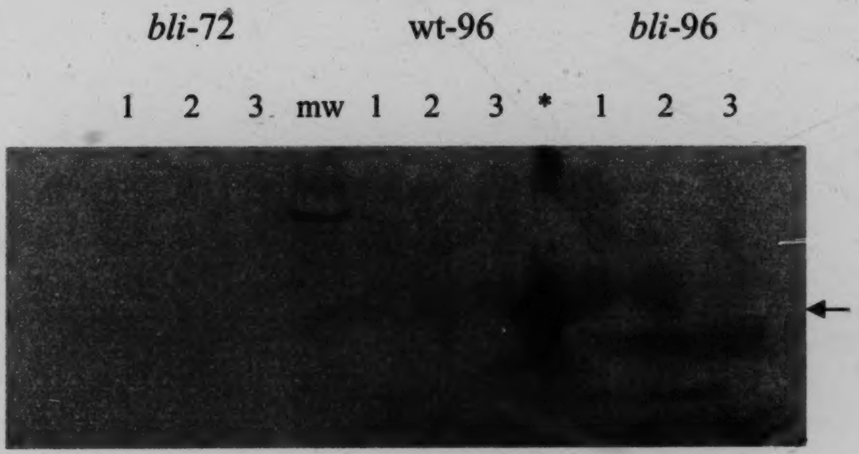
To determine if the detection limit was the reason for the inability to detect BLIP activity in samples where negative results were obtained from Bactopenase inhibition assays, the culture supernatants were dialyzed and concentrated. Dialysis would also remove components from the media or culture supernatants such as clavulanic acid that interfered with the assay. The culture supernatants were dialyzed three times against 10 mM phosphate buffer pH 7, concentrated to 1/5 original volume and reassayed. The results are shown in the "dialyzed and concentrated" column (Table IX). The samples that had shown marginal activity prior to dialysis had definite BLIP activity after dialysis and concentration although the increase in activity, which ranged from 1 to 4 fold that of the original sample, was not in proportion to the theoretical five fold increase in protein concentration. Samples from which BLIP activity could be detected were those in which GSPG trace elements were present in the media. BLIP activity was never detected in samples 9 and 11, most likely due to the detection limit of the enzyme assay. All samples that did not show BLIP activity prior to dialysis also did not show activity after dialysis and concentration. These samples included culture supernatants from media 2, 4, 6, 8, 10 and 12, which contain SA trace elements as well as samples 9 and 11 which contain GSPG trace elements. The inability to detect BLIP activity by enzyme assays in samples 2, 8, 10, and 12 where a mature BLIP signal could be seen by western analysis could also be attributed to the limit of detection of the Bactopenase inhibition assay.

VI.3 WESTERN ANALYSIS OF BLIP IN SOY FERMENTATION BROTH.

Since BLIP is produced by wild type *S. clavuligerus* cultures grown in TSBS, a medium that does not support high levels of clavulanic acid or clavam production, it was of interest to determine if BLIP was also produced in soy medium, also a complex medium which does support high level production of clavulanic acid and clavams. Soy cultures of wild type *S. clavuligerus* and the *bli* mutant were grown as described in Materials and Methods. Cultures were harvested at 48, 72 and 96 hour time points. Proteins from culture supernatants were precipitated and separated on 12% SDS-PAGE gels. 100 µl of culture supernatants, each containing ca. 80-190 µg of proteins, were run per lane. Purified BLIP was included as the positive control while supernatants from the *bli* mutant cultures were the negative controls. The gels were used to prepare western blots and the membranes hybridized with BLIP antibodies. The results, shown in Figure 34, show that no BLIP protein was present at any point in the time course in wild type culture soy medium supernatants, a surprising result as even in SA culture supernatants where no BLIP activity could be detected by enzyme inhibition assays, a trace amount of BLIP could be detected by western analysis. It is interesting that no BLIP was seen on western hybridization of soy culture supernatants since soy medium consisted of mostly soybean flour and starch while TSBS medium consisted of starch and trypticase soy broth which is a casein-soybean digest.

Figure 34. Western analysis for the presence of the BLIP protein in triplicate culture supernatants of wild type *S. clavuligerus* and the *bli* mutant grown in soy medium to 48, 72, 96 hours. Culture supernatants containing 80-190 μg of total proteins were precipitated with an equal volume of methanol and separated on a 12 % SDS-PAGE. Proteins were blotted onto PVDF membranes and the presence of the BLIP proteins was determined by immunodetection using BLIP antibodies. The lanes labelled as mw contained protein size standards while the lanes labelled with the asterisk contained 1 μg of purified BLIP protein. The arrow indicates the band corresponding to the BLIP protein.


wt-48
bli-48
wt-72
1
2
3
mw
1
2
3
*
1
2
3


bli-72
wt-96
bli-96
1
2
3
mw
1
2
3
*
1
2
3

VI.4 COMPARISON OF THE PRODUCTION OF BLIP, CLAVULANIC ACID AND CLAVAMS IN VARIOUS MEDIA.

Culture supernatants from wild type *S. clavuligerus* grown in SA, GSPG, TSBS and Soy media were subjected to HPLC analysis for the presence of clavulanic acid and clavams. Table X compares the various media studied with respect to the presence or absence of BLIP, clavulanic acid and clavams. BLIP was detected by western analysis; clavulanic acid and clavams were detected by reverse phase HPLC analysis. Results show that although BLIP is produced in abundance in TSBS, it is not produced at all in soy medium. Clavams, on the other hand, are produced in soy but not TSBS. In SA culture filtrate, clavulanic acid and clavams were seen; BLIP activity however could not be detected by Bactopenase inhibition although enough BLIP proteins were produced to be seen by western analysis. (The detection of clavams in SA culture supernatants was interesting in that there has not been a consensus on whether clavam production occurs in SA cultures. Evidence for the presence of clavams in SA culture supernatants is discussed below.) In GSPG culture supernatants, BLIP was present but clavams were difficult to detect. Although they are not mutually exclusive, these results suggest an inverse relationship in the production of BLIP and clavams and echo the finding that the *bli* mutant and the *bli/claR* double mutant showed elevated levels of clavam production when compared to wild type *S. clavuligerus*.

When culture supernatants of wild type *S. clavuligerus* and the *bli* mutant grown in SA medium were subjected to HPLC analysis for comparison of clavulanic acid production, peaks were seen where clavam-2-carboxylate and hydroxymethyl clavam would be expected. Figure 35 shows HPLC traces of culture supernatants of wild *type S. clavuligerus* and the *bli* mutant grown in SA medium for 24, 48, 72 and 96 hours. It is interesting also that at all time points, clavam levels appear greater in the *bli* mutant than in wild type. In comparison with wild type *S. clavuligerus*, the elevated level of clavam production in a *bli* mutant seen in soy culture supernatants (Results Section III) was also evident in SA grown cultures.

VI.5 WESTERN ANALYSIS OF *ccaR* CULTURE SUPERNATANTS FOR THE PRODUCTION OF BLIP.

Table X. Comparison of the production of BLIP, clavulanic acid and clavams in various media.

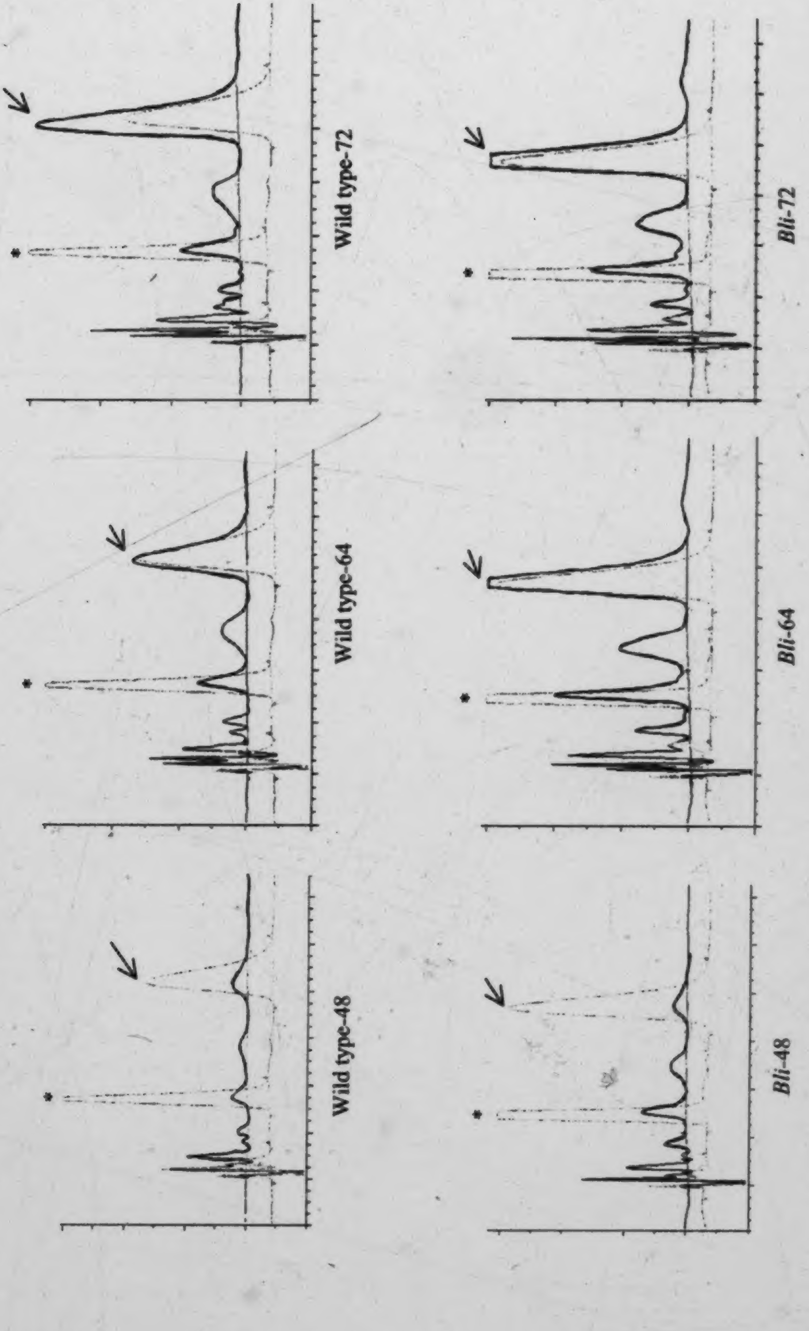
	TSBS	Soy	SA	GSPG
BLIP[a]	++++	-	+	++
Clavulanic acid[b]	+	++++	++++	+++
clavams[b]	-	+++	+/-[c]	-

[a] Culture supernatants from wild type *S. clavuligerus* grown in TSBS, soy, SA and GSPG were analysed for BLIP by western immunodetection assays. Cultures were grown as described in Materials and Methods. Supernatants from 64, 72 and 96 hour cultures were used for these assays.

[b] Clavulanic acid and clavams were analysed by reverse phase HPLC.

[c] The production of clavams in SA media was not always consistently seen.

Figure 35. HPLC analysis of SA culture supernatants of wild type *S. clavuligerus* and the *bli* mutant for β-lactam products. Culture supernatants were harvested at 48, 64 and 72 hour and 100 µl was analysed by reverse phrase HPLC. Clavulanic acid and clavam-2-carboxylate were identified by comparison to authentic standards. Traces obtained for culture supernatants are represented by the darker curves while the standards, superimposed on the same plots, were represented by the dotted faint curves. Clavam-2-carboxylate peaks are indicated by the asterisks while clavulanic acid peaks are indicated by the arrow. Hydroxymethyl clavam is presumed to be the peak that runs between clavam-2-carboxylate and clavulanic acid as standards are not available. Although not shown, the X and Y axis of all graphs have identical scales.


Wild type-72
Wild type-64
Wild type-48
Bli-72
Bli-64
Bli-48
*
*
*
*
*
*

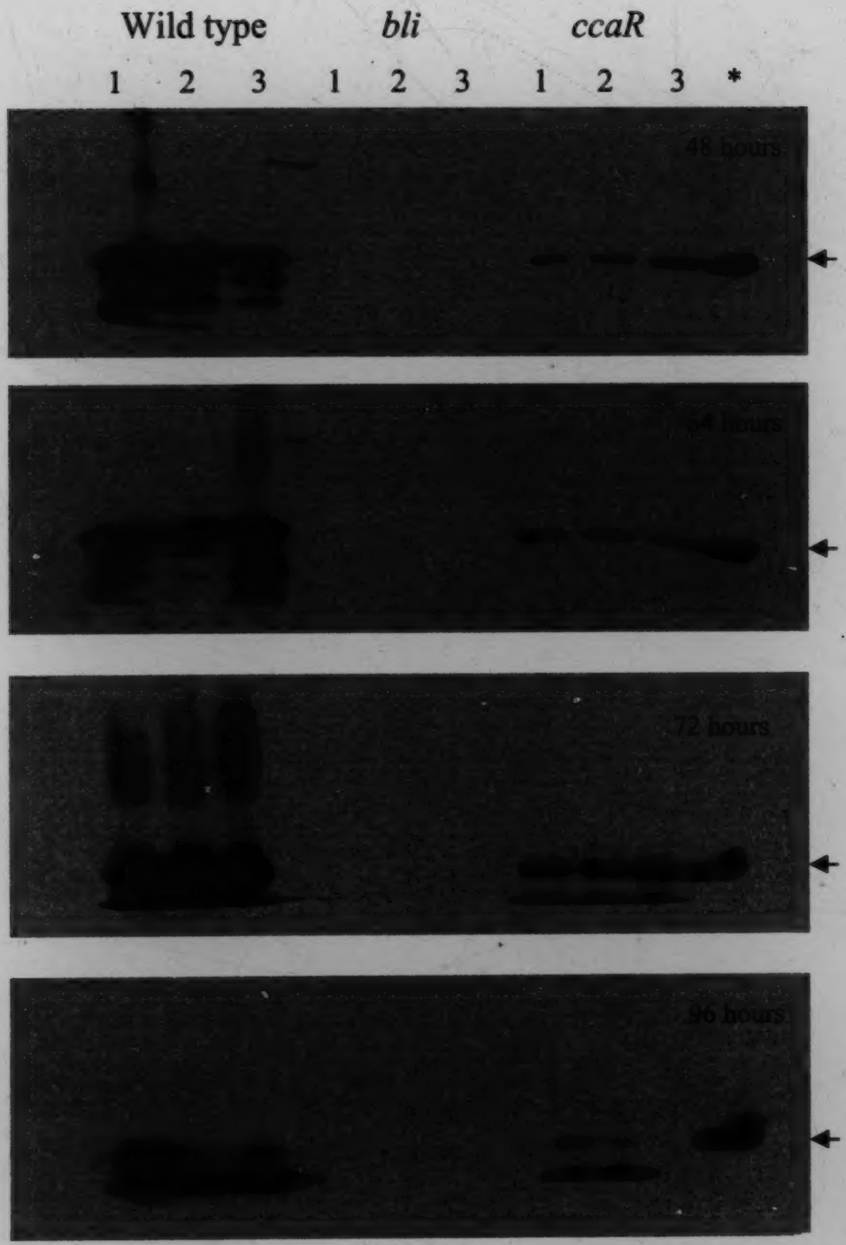
Since the *bli* gene is shown to be located near genes involved in the biosynthesis of the non-clavulanic acid clavams, such as clavam-2-carboxylate and 2 hydroxymethyl clavam, and since the *bli* mutant showed elevated levels of clavam production, it was of interest to determine if the *ccaR* mutant, a transcriptional activator mutant that is defective in production of all β-lactams, produced BLIP. Triplicate TSBS cultures of wild type *S. clavuligerus*, the *bli* and *ccaR* mutants were grown as described in Materials and Methods. The cultures were harvested at 48, 64, 72, and 96 hours. OD_{600} values for each cultures were determined at time of harvest and are summarized in Table XI. To compare the production of BLIP in the various cultures, 100 μl of supernatant from each culture was analysed for the presence of BLIP by western hybridization. The results in Figure 36 show that although a signal corresponding to the BLIP protein was present at all time points in all *ccaR* culture supernatants this signal was uniformly less than that seen in wild type samples. (The extra bands present in the wild type samples were presumably degradation products of the BLIP protein.) The difference in BLIP levels between wild type and the *ccaR* mutant was not a result of growth differences as shown by similarities in OD_{600} values. Furthermore, protein assays of the culture supernatants analysed in Figure 36 showed that, at all four time points, the amounts of proteins per μl in the culture supernatants of the *ccaR* mutant cultures were equal to or higher than those of the wild type cultures (data not shown).

Table XI. Optical densities at 600 nm of 48, 64, 72 and 96 hour cultures[a] of wild type *S. clavuligerus*, *bli* and *ccaR* mutants grown in TSBS medium.

Strains	48 hours			64 hours			72 hours			96 hours		
wild type	16	13	15	17	19	19	17	15	17	14	15	13
bli	14	17	15	18	16	16	17	17	18	15	14	14
ccaR	18	13	17	17	18	14	16	17	17	15	15	15

[a] Triplicate cultures of each strain were grown to the respective time point at which time the OD_{600} of each culture was determined and the supernatants were analysed by western hybridization for the presence of the BLIP protein.

Figure 36. Western analysis of triplicate TSBS culture supernatants of wild type *S. clavuligerus*, the *bli* mutant and the *ccaR* mutant for the presence of the BLIP protein. Cultures were harvested 48, 64, 72 and 96 hours. Proteins from 100 µl of culture supernatants, (ca. 22-70 µg), were separated on a 12 % SDS-PAGE, blotted onto PVDF membranes and hybridized with BLIP antibodies. The last lane labeled with the asterisk contained 1 µg of purified BLIP protein. The arrow indicates the band corresponding to the BLIP protein.


Wild type
bli
ccaR
1 2 3 1 2 3 1 2 3 *
48 hours
64 hours
72 hours
96 hours

VII. DISCUSSION

The production of a protein having inhibitory activity against β-lactamase is known to take place in two *Streptomyces* species, *S. clavuligerus* (Doran *et al.*, 1990) and *S. exfoliatus* (Kim, *et al.*, 1994). With the exceptions that the proteins are exocellular and the common β-lactamase inhibitory activity, there are few similarities between the *S. clavuligerus* BLIP (β-lactamase inhibitory protein) and BLIP-I and BLIP-II of *S. exfoliatus*. In both cases, no work has been done with respect to determining the importance of BLIP, in the context of the producers' life cycles or physiology. In this thesis, the function of BLIP in the *S. clavuligerus* life cycle and β-lactam production was studied. Four experimental approaches were used to elucidate the native function of BLIP. Mutants defective in BLIP production were created by gene disruption and homologous replacement. Genetic mapping experiments were carried out with the aim of determining whether the *bli* gene is linked to β-lactam genes. Sequencing of the regions adjacent to the *bli* gene was done to determine what types of genes are in the vicinity of *bli* in an attempt to identify the cellular context in which BLIP functions. And lastly, nutritional studies were performed to further understand how BLIP production is regulated and whether its expression coincides with any of the β-lactam products of *S. clavuligerus*.

In the gene disruption experiments, two BLIP nonproducers were created. A *bli* mutant was created by disruption of the *bli* gene in wild type *S. clavuligerus*. The *bli/claR* double mutant, a clavulanic acid/BLIP nonproducer, was created by disruption of the *bli* gene in an existing *claR* mutant, a clavulanic acid nonproducer. The seven *bli* single mutants and the one *bli/claR* double mutant that were isolated all showed the expected negative results when the corresponding culture supernatants were assayed for the presence of a proteinaceous β-lactamase inhibitory activity. This confirmed that BLIP production had been disrupted and suggested that no second BLIP activity existed in *S. clavuligerus*, in contrast to the scenario in *S. exfoliatus* in which two different BLIPs are produced.

Although BLIP activity was detected in culture supernatants of wild type *S. clavuligerus* grown in TSBS medium, the absence of any detectable BLIP activity in the culture supernatants of the *bli* and the *bli/claR* mutants also indicated that BLP, the BLIP-like protein of *S. clavuligerus* whose gene showed significant similarity to the *bli* gene (Perez-Llarena and Liras *et al.*, 1997), does not have β-lactamase inhibitory activity despite having 29.2 % identical amino acids and 27.6 % functionally conserved residues. (GCG/netBLAST search reported 33.8 % identity and 42.2 % similarity at the amino acid level.)

The *bli* mutant and the *bli/claR* double mutant were analysed for aberrant growth phenotypes, changes in β-lactam production and changes in resistance to β-lactams. With respect to mycelial cell structure during growth and differentiation, and sporulation, all seven single mutants were the same as the wild type strain in appearance and in their ability to undergo differentiation and sporulation. No apparent unusual growth characteristic was observed in the *bli/claR* double mutant as well which indicated that BLIP most likely does not play a required role in the growth and development of *S. clavuligerus*.

Comparison of antibiotic and clavulanic acid production showed that no apparent differences were found between wild type *S. clavuligerus* and the *bli* mutant. The double mutant, due to the *claR* disruption, did not produce clavulanic acid; however its level of cephamycin production was not affected by the *bli* disruption when the *claR* single mutant was compared to the *bli/claR* double mutant. The production of wild type levels of cephamycin by the *bli* single mutant and the *bli/claR* double mutant suggests that BLIP does not play a role in the production and secretion of active antibiotic. It also shows that BLIP does not play a required role in protection of *S. clavuligerus* against its own antibacterial products. If BLIP was important in self-protection of *S. clavuligerus* against its own antimicrobial products, the isolation of a *bli* mutant displaying a wild type level of cephamycin production would not be likely; instead, only cephamycin nonproducing *bli* mutants could be isolated. The disruption of the *bli* gene had little effect on most aspects of *S. clavuligerus* development or production of secondary metabolites with the exception of the production of clavams, the β-lactam compounds produced by *S. clavuligerus* that have antifungal activity but weak antibacterial activity in comparison with the cephamycins and penicillins. Both the *bli* mutant and the *bli/claR* double mutant showed transient elevated levels of clavam-2 carboxylate production at the 72 hour time point when compared to wild type and the *claR* mutant. Levels of hydroxymethyl clavams were unaffected in the *bli/claR* double mutant when compared to the *claR* single mutant; however, the *bli* mutant showed elevated levels of this clavam at 72 and 96 hours of growth. Elevated levels of clavam production were also seen in nutritional studies in which SA culture supernatants of the *bli* mutant and of wild type *S. clavuligerus* were compared by HPLC. This transient effect in clavam production in the *bli* single and *bli/claR* double mutants is the first experimental evidence that BLIP and clavams may be linked. It is difficult to visualize a role for BLIP in the production or secretion of the clavams since the effect seems to be transient.

The resistance of the mutants to Penicillin G in liquid cultures and surface cultures was studied. No significant differences were seen among wild type, the *claR* mutant and the *bli* and *bli/claR* double mutant strains. For surface culture studies, both germinated and ungerminated mycelia were studied. Although BLIP has activity against certain β-lactamases, it does not seem to contribute to the innate resistance of *S. clavuligerus* to the β-lactam Penicillin G either in surface cell cultures or in liquid cultures. This finding was not unexpected, as the high resistance of *S. clavuligerus* cultures to β-lactam antibiotics is believed to be a result of the production of PBPs having low affinity to β-lactams (Ogawara *et al.*, 1980). Although *S. clavuligerus* has been shown to possess a β-lactamase gene (Perez-Llarena *et al.*, 1997), it is less likely that the high resistance that *S. clavuligerus* displays towards these β-lactam antibiotics is a result of the presence of the β-lactamase as this activity has been difficult to detect. This idea is supported by the finding that in general, no relationship exists between minimum inhibitory concentrations of benzylpenicillin and β-lactamase production (Ogarawa, 1978).

Although difficult, the successful isolation of a *bli/claR* double mutant suggests that β-lactamase inhibitors are not essential in the *S. clavuligerus* lifecycle. Since only a single *bli/claR* double mutant was isolated despite extensive screening (60 000 colonies), the question arises whether the double mutant harbors a third mutation which then allows for the β-lactamase inhibitor negative state and whether the isolated "double" mutant is representative of a BLIP and clavulanic acid negative strain. When the frequency of isolating the single mutant (1 in 2 000) is compared to that of isolating the double mutant (1 in 60 000), it is clear that a significant difference exists in the rate of homologous double crossover events at the *bli* locus occurring during creation of the *bli* single mutant when compared to the creation of the the *bli/claR* double mutant. Since the plasmid construct used in the disruption event was the same in the creation of the single and double mutants, the difference in the frequency of crossover events was not due to length of homologous sequences since they were identical. If β-lactamase inhibitors are, in some unknown way, essential in *S. clavuligerus* biology, then it is possible that a third mutation was required for generating a viable double mutant. Since neither a *bli* nor a *claR* single mutant shows abnormal growth characteristics, it is unlikely that β-lactamase inhibitors are essential for the normal functioning of *S. clavuligerus* and unlikely that a third mutation related to the function of β-lactamase inhibitors is required to establish the β-lactamase inhibitor negative state. Paradkar *et al.*, (1998) showed that the *claR* gene encodes a transcriptional regulator that activates the transcription of the late genes involved in clavulanic acid biosynthesis. Inactivation of the *claR* gene appears to affect only clavulanic acid production.) Furthermore, all aspects of *S. clavuligerus* growth, differentiation and secondary metabolite production, with the exception of the transient increase in clavam production which was also seen in the single *bli* mutant, appear normal.

Instead, it was likely that the difference in frequency of homologous double crossovers at the *bli* locus during the creation of the *bli* and *bli/claR* mutants lies in the host background, wild type compared to *claR* mutant. The *claR* mutant used in the creation of the *bli/claR* double mutant was a single clone isolated during the disruption of the *claR* gene and differed from the wild type strain in that it had already undergone one round of protoplast formation. Since protoplast formation can be mutagenic (S.E. Jensen, personal communication) it is possible that the clone containing the disrupted *claR* gene was somewhat impaired in homologous recombination, the change occurring either during the first protoplasting event or the second event during the creation of the *bli* disruption. The disruption of normal recombination machinery that is being postulated is a mutagenic event unrelated to the function of β-lactamase inhibitors in *S. clavuligerus* and is not required to achieve the β-lactamase inhibitor negative state in the *bli/claR* double mutant. This theory is proposed to explain the difficulty in isolating the double mutant and can be tested by the introduction of a different homologous recombination locus, (*geneX*), to create a *claR/geneX* double mutant.

The fact that the *bli/claR* double mutant exhibited normal growth characteristics, morphology and cephamycin production, as did the *bli* mutant, lends credibility to the belief that the isolated *bli/claR* double mutant is likely representative of a strain defective in clavulanic acid and BLIP. The similarity of the *bli* and *bli/claR* mutants with respect to elevated clavam production in soy medium further supports this conclusion. In terms of elucidating the function of BLIP, although a transient increase in clavam production in soy medium was seen in late growth stage cultures of BLIP nonproducing mutants compared to wild type cultures, a later nutritional studies experiment showed that BLIP itself is not produced in soy medium by wild type *S. clavuligerus*. Therefore it is not clear then how the disruption of the *bli* gene affects the level of clavam production or what role BLIP might play in the production or secretion of clavams given that the wild type culture does not produce BLIP in soy medium which supports high levels of clavam production. Again, the absence of BLIP in wild type *S. clavuligerus* soy cultures underscores the idea that the production of clavams seems to correlate with the absence of BLIP.

A possible explanation for the absence of any other phenotypic change in the *bli* mutants is the idea that BLP could compensate for the absence of BLIP. Although BLIP assays of the culture supernatants of the *bli* and *bli/claR* mutants have shown that *S. clavuligerus* does not produce a second protein having β-lactamase inhibitory activity, it is possible that BLP may compensate for the *bli* disruption as the cellular function of BLIP may not be that of β-lactamase inhibitor. In addition to the significant percentage of identical and functionally conserved residues in the primary sequence, a comparison of computer modelling predictions of the BLP structure with the three dimensional structure of BLIP obtained by X-ray crystallography (Strynaka *et al.*, 1994) shows that the two proteins are similar (N. Strynadka, personal communication). There is no evidence up to this point to support the theory that BLP and BLIP are functionally complementary; however, the creation and characterization of a BLIP and BLP double mutant would address this question.

In addition to the transient increase in clavam production in BLIP nonproducing mutants, there is some evidence that the linkage of BLIP and clavams is also reflected at the genetic level. Southern hybridization and PCR experiments with cosmids containing clavam genes demonstrate that the *bli* gene and clavam biosynthetic genes are physically linked on the chromosome. When the cosmid NL1D1, which was isolated by Southern hybridization with an oligonucleotide designed to hybridize to a region in the *cas1* gene, was used as the template DNA in PCR reactions designed to amplify the N-terminal half of the *bli* gene, a product was obtained and confirmed to be part of the *bli* gene by sequencing. Southern hybridization experiments in which NL1D1 was probed with the *bli* gene showed that *bli* was present on a 5 kilobase *Nco* I fragment. This fragment was also present on pBIP, a cosmid from which the *bli* gene was initially cloned (Doran *et al.*, 1990), as well as in *Nco* I digests of genomic DNA. Attempts to identify larger fragments containing the *bli* gene that are conserved in NL1D1, pBIP and genomic DNA have not been successful. Southern hybridization results obtained showed that in NL1D1, regions beyond the 5 kilobase *Nco* I fragment have undergone rearrangements and perhaps deletions as well. Comparison of NL1D1 with genomic DNA has shown that the 9 kb *Cla* I/*Bgl* II fragment containing the *bli* gene has been rearranged in NL1D1. The exact distance and orientation of the *bli* gene with respect to the *cas1* gene are yet unknown due to the structural instability of NL1D1.

The stable cosmid p53 derived from NL1D1 did not yield a product when it was used as the DNA template in PCR amplification of the half *bli* gene. Agarose gel analysis of the insert in NL1D1 showed that the NL1D1 cosmid preparation contained at least three different clones. Comparison with the p53 insert showed that the p53 insert corresponded to the largest insert in NL1D1; however, Southern hybridization experiments showed that although the largest insert in NL1D1 hybridized to the *bli* probe, the largest insert in p53 did not, unless subjected to significantly longer exposure time. Furthermore, the proportion of NL1D1 cosmid DNA that hybridized to the *bli* probe was found to be significantly less than the proportion that hybridized to the probes corresponding to the end fragments of p53 although similar amounts of DNA were used. This shows that even the original NL1D1 preparation was a mixed population of cosmids, only a proportion of which still contained the *bli* gene.

Comparison of the insert in p53 and in NL1D1 cosmid preparations by Southern hybridization showed that the end regions of the two inserts are similar, these being the 4 kilobase *Bgl* II/*Xba* I left fragment and the 3.3 kilobase *Bam* HI/*Hind* III right end fragments. Since NL1D1 was a mixed population of cosmids, it is possible that the original NL1D1 insert has different ends than the p53 insert and that the apparent similarity is an indication that the NL1D1 preparation had deleted and rearranged to a point where most of the cosmids in the preparation are actually p53. An alternative explanation is that the ends of the original NL1D1 insert are the same as those of the p53 insert which then means that whatever rearrangements and deletions had taken place within the NL1D1 insert, resulting in the loss of *bli* sequences, these did not encompass the end fragments and had to have taken place within an internal region of the insert. Based on these two possibilities, the *bli* gene can be located in two possible places relative to the *cas1* gene.

Based on the premise that the end regions of the insert in NL1D1 and p53 are conserved and that the region of instability is internal within the NL1D1/p53 insert, the *bli* gene must then be located right of the 9 kb *Sac* I fragment of the *cas1* region for the following reasons. Since the 7 kilobase fragment and 9.5 kilobase fragment which encompass the *cas1* region are intact in the p53 insert (R. H. Mosher and C. Anders, unpublished result), the region of rearrangements and deletions would then be either in the region right or left of the *cas1* area but not including the actual end fragments. Cosmid hybridizations showed that the *bli* gene is not located in sequences between the *cas1* region and the left probe fragment. Furthermore, the *bli* probe and the Bgl-1.6 probe both hybridized to the same fragment in two different NL1D1 cosmid digests. This fragment corresponds to the *cas1* region and all of the insert to the right of this *cas1* region in a *Hind* III digest, or just the portion of the insert that is to the right of the *cas1* region in a *Sac* I digest.

Attempts to further map the *bli* gene within this area by Southern hybridization confirmed that this internal region had undergone rearrangements and deletions both in the p53 insert and in the NL1D1 insert. When *Sac* I/*Cla* I digests of NL1D1 and cosmid p53 DNA were probed with the Bgl-1.6 probe, hybridizing bands obtained were different between the two cosmids. When the same digests were probed with the *bli* probe, hybridizing bands obtained in NL1D1 and cosmid p53 were also different. In all cases, hybridizing bands in the cosmid DNA were smaller than in genomic DNA. Rearrangement at the *bli* locus was further demonstrated when the *Bgl* II/*Cla* I digests of pBIP and NL1D1 DNA both showed different hybridization patterns with the *bli* probe.

The demonstration that the region in the vicinity of the *bli* gene as well as the region in the vicinity of the Bgl-1.6 fragment have undergone rearrangments in NL1D1 does not rule out the possibility that the *bli* gene is located on DNA sequences beyond the right end of the p53 insert. This would be the case if the second hypothesis, that the apparent similarity in the end fragments of NL1D1 and p53 is a result of the high proportion of p53 form in the NL1D1 preparation, is true. If so, then in actuality, the two inserts have different end fragments and the *bli* gene might then be located on DNA sequences beyond the right end of the p53 insert.

The *bli* gene cannot be located beyond the left end of the insert based on the finding that the *bli* gene hybridizes with the Bgl-1.6 probe as well as R-3.3, the right end fragment of the p53 insert, in a *Hind* III and a *Sac* I digest. If *bli* is located beyond the right end of the p53 insert, genomic hybridizations show that it must be at least 3.7 kilobases beyond the right end of the insert. Since the insert in cosmid p53 is approximately 45 kilobases, it is unlikely that the insert in NL1D1 is significantly larger than this due to packaging limitations.

Instability appears to be the common theme in all *bli* containing cosmids including pBIP. Evidence in support of deletions and rearrangements in the pBIP cosmid included the following. When the region on either side of the *bli* gene in pBIP was sequenced, at least one apparent deletion event downstream of the *bli* gene was seen to have taken place. This deletion, believed to be approximately 100-200 basepairs, occurred within a gene showing high homology to ABC cassette transporter genes. The size of the deletion cannot be much larger than 100-200 basepairs since no difference in the size of the *Cla* I/*Bgl* II fragment in pBIP and genomic DNA was detected in Southern hybridization experiments discussed above.

This instability in the area encompassing the *bli* locus in all *bli* containing cosmids, NL1D1, p53 and pBIP, accounts for the difficulty in mapping the *bli* gene using isolated cosmids such as NL1D1 and p53. Further refinement in mapping the *bli* locus using existing cosmids is not likely to be helpful as deletions and rearrangements are unpredictable and unlikely to be a single event since even the original NL1D1 preparation contained a mixture of at least three different clones. The isolation of p53, the stable derivative of NL1D1, was difficult (A. Wong, A. Paradkar and S. E. Jensen, unpublished work) as more often than not, smaller deleted derivatives were obtained. Furthermore, even in the comparatively stable pBIP insert, at least one and presumably two deletion events are known to have taken place. The first deletion event is the deletion of the 100-200 basepair segment in the ABC transporter gene while the second event would have taken place in the original pBIP insert which must have been 20-25 kb since the current 13.5 kb pBIP insert would not have been packaged. Although data exist to support the conclusions that the *bli* locus is downstream of the *cas1* gene and is also within 30 kb of genes involved in clavam biosynthesis, further work using genomic DNA is required to map its location definitively with respect to the *cas1* gene as it is unlikely that cosmid clones would be useful due to the instability problem. One experimental approach that would circumvent the problem of cosmid instability is the use of CHEF (clamped homogeneous electric fields) gel electrophoresis as larger fragments of genomic DNA could be separated and analysed.

Additional attempts at elucidating the function of BLIP involved sequencing the region in the vicinity of the *bli* gene. An open reading frame had previously been discovered by A. Paradkar upstream of the *bli* gene (unpublished result). The ORF, called *bliup1*, is divergent to the *bli* gene and shows no homology to any known proteins. Sequencing results obtained for a 1 kb region beyond the *bliup1* gene showed no detectable ORFs. The absence of coding sequences in a 1 kb region is an unusual feature of *S. clavuligerus* DNA sequences (S. E. Jensen, personal communication) as *S.*

clavuligerus DNA generally show a high density of ORFs, some of which are overlapping, and separated by small intergenic sequences.

Results of sequencing regions downstream of the *bli* gene showed several ORFs, the first two (*atp-A* and *atp-B*) having high similarity to ATP-binding proteins. The *atp-A* and *atp-B* ORFs each contain one of the two Walker motifs believed to be involved in nucleotide binding. An unresolved frameshift is seen at a position 200 basepairs into the 5' end of the sequence resulting in Walker A motif being in frame 1 and Walker B motif and the remaining sequence in frame 3. Alignments with other ABC transporter proteins also show that the coding region of conserved residues in the two motifs overlap within the sequence. This indicates that the deletion involved a region of 84 to 183 bases encoding a 28 to 61 amino acid segment that is part of a loop joining Walker A and B motifs. The two absolutely conserved K and D residues in Walker A and B motifs respectively of other known *Streptomyces* ABC transporters range from 116-150 amino acids apart (Mendez *et al.*, 1998) while the same residues in predicted amino acid sequence of this ABC transporter protein are only 88 amino acids apart. It is highly unlikely that this frame shift is a result of sequencing errors as both strands were sequenced twice.

For the above reason, I believe that both ORFs are part of one large gene that has undergone deletion(s) in a center segment. ABC transporters generally consist of two ATP-binding domains and two membrane spanning domains (MSDs) all of which can be in separate peptides or in various permutations of ATP-binding and MSDs including two ATP-binding domains on one peptide and two MSDs on another; or one ATP-binding domain fused with one MSD forming one peptide. The actual ATP-hydrolysis-dependent transporter is generally composed of two of each domain, however. For this reason, it is not clear if the deleted segment encodes a loop joining the Walker A element in *atp-A* and the Walker B element in the *atp-B* ORF or if the region deleted has two Walker motifs: a Walker B element to match the Walker A element observed in the *atp-A* ORF and a Walker A element to match with the Walker B element identified in the *atp-B* ORF.

A third ORF was identified immediately downstream of the *atp-A/B* ORFs. This ORF shows homology to no known proteins; however, at least six hydrophobic putative transmembrane domains can be seen by the Kyte Doolittle hydropathy analysis method (Kyte and Doolittle, 1982). It is most likely that this ORF encodes the MSDs of an ABC transporter protein whose ATP-binding domains are encoded by the *atp-A/B*

ORFs. The ORF has probably not been completely sequenced since the membrane spanning regions of ABC transporters generally consist of two domains each having six hydrophobic transmembrane segments (Higgins, 1992). It is possible that only half the gene for this transmembrane segment has been sequenced. The lack of homology with other MSDs is not surprising; although the ATP-binding domains are highly conserved throughout the entire peptide, the MSDs of different transporters are vastly different. It is believed that the MSDs, which form a hydrophilic pore through which various substrates are transported determines substrate specificity. Since ABC transporters have been known to import or export a variety of substrates that include proteases lacking signal sequences (*Erwinia chrysanthemi*), sugars (maltose and ribose in *E. coli*), capsule polysaccharide in *E. coli* and *Neisseria* and antibiotics (daunomycin in *Streptomyces peucetius*) (Higgins, 1992), it is not surprising that the MSDs do not show a high degree of similarity among themselves. The lack of homology of the identified ORF downstream of *atp-A/B* with any known proteins is consistent with the finding that little sequence conservation exists among the MSDs of bacterial ABC exporters (Fath *et al.*, 1993).

The finding of an ABC transporter gene immediately downstream of the *bli* gene suggests that perhaps BLIP plays a role in secretion. A number of antibiotic producing actinomycetes contain ABC transporter genes that will confer resistance to a sensitive host when cloned and heterologously expressed (Mendez *et al.*, 1998). The ABC exporter mediates export of the antibiotic from the cell by an active process involving ATP hydrolysis. It is possible that BLIP functions as an accessory protein in an ABC dependent secretion system since accessory factors are always found linked to the ABC exporter gene (Fath *et al.*, 1993). Accessory factors in Gram negative organisms are believed to aid in export through the inner and outer membrane by spanning the periplasm, connecting the two membranes. It is not clear what role the accessory protein plays in Gram positive organisms such as *S. clavuligerus;* however, accessory factors linked to ABC exporter genes of the Gram positive bacteria such as *Bacillus subtilis*, *Streptococcus pneumoniae* and *Lactococcus lactis* have been described (Fath *et al.*, 1993). Since only a transient effect on the level of clavam production was observed in the *bli* mutants while no change in cephamycin or clavulanic acid production was seen, if BLIP functions as an accessory protein, it is possible that either the accessory protein plays a minor role in secretion or that its absence was compensated for by the BLP protein. The fact that both BLIP and BLP are internally dimeric is interesting in that ABC transporters consist of two ATP-binding domains and two MSDs as stated above.

If BLIP functions as an accessory in an ABC transport system, its β-lactamase inhibitory activity would then be coincidental. It is also possible that the juxtaposition of the *bli* gene with ORFs encoding ABC transporter proteins is also coincidental in which case BLIP would not have transport functions.

Remaining work on BLIP involved the study of BLIP production under different nutritional conditions. Preliminary work showed that BLIP is produced in the complex medium TSBS and in the defined medium GSPG. No BLIP activity was detected by Bactopenase inhibition assays of SA culture supernatants. Initial experiments on BLIP production were approached with the aim of identifying the difference between GSPG and SA that would account for BLIP production in GSPG and not SA, since both are defined media. BLIP activity was determined by measuring the β-lactamase inhibitory activity of culture supernatants with and without heat inactivation. Comparison of GSPG and SA medium compositions showed that the two media differed in carbon and nitrogen sources, buffer type and amounts of trace elements. Various derivatives of GSPG and SA media were made and tested for their ability to support BLIP production.

Results of β-lactamase inhibition assays showed that although in SA, clavulanic acid was stable during the heat inactivation step that is required to distinguish between inhibition due to BLIP, in GSPG culture supernatants, clavulanic acid was unstable to the heat inactivation step. Western immunodetection was then developed for determining BLIP activity. Western hybridization of SA culture supernatants showed that BLIP is present although BLIP activity in SA was never detected by β-lactamase inhibition assay. It is possible that the amount of BLIP in SA culture supernatants was below the detection limit of the β-lactamase inhibition assay. Although BLIP can be detected in SA culture supernatants by western analysis, the production of BLIP was most efficient under conditions of higher trace element concentrations. Furthermore, the production of mature BLIP was found to be more dependent on the amount of trace elements than on carbon and nitrogen sources or buffer type, with lowest levels of mature BLIP proteins being produced in conditions of low ionic strength such as SA medium.

Since BLIP is produced in abundance in the complex medium TSBS and less so in the defined media GSPG and SA, BLIP production in soy medium, a second complex medium was analysed. Western hybridization results showed that no trace of BLIP could be detected in soy culture supernatants at any time in the growth phase of the culture. This is interesting in that TSBS and soy media are both complex media, TSBS is trypticase soy (soybean-casein digest medium) and starch while soy medium contained soybean flour, starch and 2% $FeSO_4 \cdot 7H_2O$. This result is interesting because soy medium is the only medium in which clavam production can be consistently detected.

Comparison of the pattern of production of BLIP with the other β-lactam products of *S. clavuligerus* grown in various media showed no informative relationships between their pattern of production with the exception that *ccaR* , the transcriptional activator mutant that is defective in production of all classes of β-lactam compounds, also showed a much lower amount of BLIP in the culture supernatants throughout its time course when compared to wild type *S. clavuligerus*.

Despite the work discussed here, the exact function of BLIP remains elusive. An alternative and more speculative theory for the function of BLIP is that it is simply a protease inhibitor. Its inhibitory activity against β-lactamase, a serine active enzyme, places it in the class of serine protease inhibitors. Both trypsin type and metallo-type exocellular proteases have been detected in *S. clavuligerus* (Jeffries *et al.*, 1980 and Buckley *et al.*, 1981) however BLIP does not seem to have an effect on these enzymes (Jensen, personal communication).

An interesting example of a proteinaceous protease inhibitor is the inhibitor of proteases B and C of *Erwinia chrysanthemi* (Wandersman, 1989). The inhibitor in *E. chrysanthemi* is a small heat stable periplasmic protein believed to bind to proteases B and C. The gene for this inhibitor is also located on a DNA fragment containing genes involved in secretion of proteases B and C. Interestingly, proteases B and C do not have N-terminal signal peptides and their secretion system is analogous to that of α-haemolysin, the first ABC export system identified in prokaryotes (Fath *et al.*, 1993).

VIII. BIBLIOGRAPHY

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